IDENTIFICATION, EXPLOITATION AND MANIPULATION OF BRCA1-DEPENDENT DNA DOUBLE-STRAND BREAK AND INTERSTRAND CROSSLINK REPAIR IN BREAST AND OVARIAN CANCER THERAPY

By

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Abstract

Expression of functional breast cancer susceptibility gene 1 (BRCA1) in human cancers is associated with resistance to platinum-based chemotherapeutics and poly(ADP-ribose) polymerase (PARP) inhibitors. BRCA1 is a nuclear phosphoprotein with broad tumor suppressor activities that, among other functions, is critical for resolving double-strand DNA breaks (DSBs) and interstrand crosslinks (ICLs) by homologous recombination (HR). *In vitro,* animal and human clinical data have demonstrated that BRCA1-deficient cancers are highly sensitive to ICL-inducing alkylative chemotherapeutic agents, are amenable to synthetic lethal approaches which exploit defects in DSB/ICL repair (e.g., PARP inhibitors), and are generally associated with more favorable responses to anti-neoplastic therapy and improved survival. Conversely, high expression of wild-type BRCA1 in a number of cancers, as well as frame-restoring intragenic mutations in *BRCA1* mutant ovarian cancers, is associated with therapeutic resistance and poor prognosis. Accordingly, there has been much interest in identifying, exploiting and manipulating DSB/ICL repair capacity to restore or enhance sensitivity to cancer therapeutics. In this study, we demonstrate that the heat shock protein 90 (HSP90) inhibitor 17-allylamino-17-demethoxygeldanamycin (17-AAG (Tanespimycin)), which is currently in Phase II/III clinical evaluation, induces BRCA1 ubiquitination and proteasomal degradation in numerous *in vitro* models. Mechanistically, we show that loss of HSP90 function completely abolishes both homologous recombination and non-homologous end joining of DSBs, that BRCA1-deficient cells are hypersensitive to 17-AAG due to enhanced replication stress and aberrant entry into mitosis, and that 17-AAG can reverse BRCA1-dependent repair-mediated resistance. Additionally, we assessed the role of *BRCA1* promoter methylation in sporadic triple-negative breast cancers (TNBCs) and identify a novel biomarker for poor response to

anthracycline regimens in human patients. In summary, we document a novel upstream HSP90-dependent regulatory point in the Fanconi anemia/BRCA DSB/ICL repair pathway, illuminate the role of BRCA1 in regulating damage-associated checkpoint and replication responses to HSP90 inhibitors, specifically identify BRCA1 as a novel, clinically relevant target for enhancing radio- and chemosensitivity in refractory and/or resistant malignancies, and identify a useful biomarker for studies of therapeutic sensitivity in human TNBCs.

Dedication

To my wife, Brianna:

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List of Abbreviations

17-AAG	17-allylamino-17-demethoxygeldanamycin
ABRA1	Abraxas (gene)
ALDH1A1	Aldehyde dehydrogenase 1 family, member A1 (gene)
ANOVA	Analysis of variance
ATM	Ataxia telangiectasia mutatated (gene)
ATP	Adenosine triphosphate
ATR	Ataxia telangiectasia and Rad3-related (gene)
AZD-2281	Olaparib
BARD1	BRCA1-associated ring domain 1 (gene)
BER	Base excision repair
BLBC	Basal-like breast cancer
BRCA1	Breast cancer susceptibility gene 1 (gene)
BRCA2	Breast cancer susceptibility gene 2 (gene) [same as FANCD1]
BRCC36	BRCA1/BRCA2-containing complex subunit 36 (gene)
BRCC45	BRCA1/BRCA2-containing complex subunit 45 (gene)
BRCT	BRCA1 C terminus domain
BRIP1	BRCA1-associated C-terminal helicase (gene) [same as FANCJ]
BSI-201	Iniparib
cCR	Clinical complete remission
CD24	Small cell lung carcinoma cluster 4 antigen (gene)
CD44	Indian blood group antigen (gene)
CD49f	Integrin α6 (gene)
CDC2	Cyclin-dependent kinase 1 (gene) [same as CDK1]
CDC25A	Cell division cycle 25 homolog A (gene)
CDC25C	Cell division cycle 25 homolog C (gene)
CDK1	Cyclin-dependent kinase 1 (gene) [same as CDC2]
CDK5	Cyclin-dependent kinase 5 (gene)
CGH	Comparitive genomic hybridization
CHK1	Checkpoint kinase 1 (gene)
CHK2	Checkpoint kinase 2 (gene)
CI	Combination index
CpG	Cytosine-phosphate-guanine dinucleotide
^{137}Cs	Cesium-137
CSC	Cancer stem cell
DAPI	4’-6-diamidino-2-phenylindole
DDN	2,3-dichloro-5,8-dihydroxy-1,4-naphthoquinone
DDR	DNA damage response
DEB	Diepoxybutane
DMSO	Dimethylsulfoxide
DN	Dominant negative
DNA	Deoxyribonucleic acid
DNA-PKcs	Protein kinase, DNA-activated, catalytic polypeptide (gene)
DSB	Double-strand break
EC	Epirubicin plus cyclophosphamide

EDTA	Ethylenediaminetetraacetic acid
EdU	5-ethynyl uridine
EOC	Epithelial ovarian cancer
EpCAM	Epithelial cell adhesion molecule (gene)
ER	Estrogen receptor
ESR1	Estrogen receptor 1 (gene)
FA	Fanconi anemia
FAAP24	Fanconi anemia-associated protein 24 (gene)
FAN1	Fanconi anemia-associated nuclease 1 (gene)
FANCA	Fanconi anemia, complementation group A (gene)
FANCB	Fanconi anemia, complementation group B (gene)
FANCC	Fanconi anemia, complementation group C (gene)
FANCD1	Fanconi anemia, complementation group D1 (gene) [same as BRCA2]
FANCD2	Fanconi anemia, complementation group D2 (gene)
FANCE	Fanconi anemia, complementation group E (gene)
FANCF	Fanconi anemia, complementation group F (gene)
FANCG	Fanconi anemia, complementation group G (gene)
FANCI	Fanconi anemia, complementation group I (gene)
FANCJ	Fanconi anemia, complementation group J (gene) [same as BRIP1]
FANCL	Fanconi anemia, complementation group L (gene)
FANCM	Fanconi anemia, complementation group M (gene)
FANCN	Fanconi anemia, complementation group N (gene) [same as PALB2]
FANCO	Fanconi anemia, complementation group O (gene) [same as RAD51C]
FANCP	Fanconi anemia, complementation group P (gene)
FFPE	Formalin-fixed paraffin-embedded
gDNA	Genomic DNA
GFP	Green fluorescent protein
GSTP1	Glutathione S-transferase pi 1 (gene)
GSK3β	Glycogen synthase kinase 3 beta (gene)
Gy	Gray
γH2AX	H2A histone family, member X (pS^{139})
H2AX	H2A histone family, member X (gene)
H3	Histone 3
HA	Hemagglutinin
HBOC	Hereditary breast and/or ovarian cancer
HER2	Avian erythroblastic leukemia viral oncogene homolog 2 (gene)
HNPCC	Hereditary non-polyposis colorectal cancer
HR	Homologous recombination
HRP	Horseradish peroxidase
HSF1	Heat shock factor 1
HSP27	Heat shock protein 27
HSP70	Heat shock protein 70
HSP90	Heat shock protein 90
ICL	Interstrand crosslink
ICLR	Interstrand crosslink repair
IP	Immunoprecipitation

IR	Ionizing radiation
IRB	Institutional review board
IRIF	Ionizing radiation-induced foci
Ku70	X-ray repair complementing defective repair 6 (XRCC6) (gene)
Ku80	X-ray repair complementing defective repair 5 (XRCC5) (gene)
LIG4	Ligase IV, DNA, ATP-dependent (gene)
LOH	Loss of heterozygosity
MAI	Mitotic activity index
MERIT40	Mediator of RAP80 interactions and tartgeting subunit of 40 kDa (gene)
MG132	N-carbobenzoxy-leucinyl-leucinyl-norleucinal
MGMT	O-6-methylguanine-DNA methyltransferase (gene)
MHF	FANCM-associated histone fold 1/2 (gene)
miRNA	MicroRNA
MLH1	*mutL* homolog 1 (gene)
MMC	Mitomycin C
MMR	Mismatch repair
MOI	Multiplicity of infection
MRE11	Meiotic recombination 11 homolog (gene)
MRN	MRE11/RAD50/NBS1
mRNA	Messenger RNA
MSH2	*mutS* homolog 2 (gene)
MSP	Methylation-specific PCR
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
NER	Nucleotide excision repair
NBR2	Neighbor of BRCA1 gene 2 (gene)
NBS1	Nijmegan breakage syndrome 1 (gene)
NHEJ	Non-homologous end joining
NLS	Nuclear localization signal
PALB2	Partner and localizer of BRCA2 (gene) [same as FANCN]
PARP	Poly(ADP)ribose polymerase
PBS	Phosphate-buffered saline
pCR	Pathologic complete remission
PCR	Polymerase chain reaction
PFI	Platinum-free interval
PI3K	Phosphatidylinositol-3 kinase
PKC	Protein kinase C
PLD	Pegylated liposomal doxorubicin
PMSF	Phenylmethylsulfonylfluoride
PR	Progesterone receptor
qRT-PCR	Quantitative real-time PCR
RAD50	DNA repair protein RAD50 (gene)
RAD51	*recA* homolog (gene)
RAP80	Ubiqutin interaction motif-containing protein 1 (gene)
RING	Really interesting new gene domain
SCE	Sister chromatid exchange

SEM	Standard error of the mean
shRNA	Short hairpin RNA
ssDNA	single-strand DNA
TAE	Tris-acetate-EDTA
TN	Triple-negative
TNBC	Triple-negative breast cancer
TP53	Tumor protein 53 (gene)
TU	Transducing units
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
XRCC4	X-ray repair complementing defective repair 4 (XRCC4) (gene)

Chapter I: Introduction

Introduction

Inherited defects in DNA repair pathways underlie numerous cancer susceptibility syndromes. Acquired defects in these same pathways are commonly seen in sporadic cancers and are responsible for the genomic instability that is a universal hallmark of cancer. Fanconi anemia (FA) and hereditary breast and/or ovarian cancer (HBOC) are two inherited syndromes whose pathogeneses stem from defects in DNA repair. At the molecular level, these disorders are fundamentally related as products of the Fanconi anemia (FANC) and breast cancer susceptibility (BRCA) genes operate in concert to repair specific types of DNA damage—indeed, *BRCA2* and *FANCD1* are the same gene. Though HBOC is responsible for a minority of all breast and ovarian cancers and FA is an exceedingly rare disease (1-5 cases per one 1 million persons) (D'Andrea, 2010), acquired defects or deficiencies in the FA/BRCA pathway are relatively common and may have profound implications for therapeutic efficacy in sporadic cancers.

Fanconi Anemia

FA is a rare recessive disorder with tremendous locus heterogeneity. Fifteen complementation groups are currently recognized and, with the exception of the *FANCB* complementation group, all are inherited in an autosomal recessive fashion (Table 1) (Wang, 2007). This disease is disproportionately seen in Ashkenazi Jewish individuals and several unique mutations in complementation groups *FANCC*, *FANCA* and *BRCA2/FANCD1* are seen in this population (Kutler and Auerbach, 2004). Congenital anomalies are diverse in FA, with short stature, developmental disability, and physical defects of the skin, limbs, head, eyes, ears, and kidneys being most common. The most troublesome early manifestation of FA is bone marrow failure,

Complementation Group	Gene Alias(s)	Mapping	Percentage of FA Patients
FANCA	*FANCH*	16q24.3	62.3
FANCB	*FAAP95*	Xp22.2	2.1
FANCC		9q22.32	13.7
FANCD1	***BRCA2***	13q12.3	2.5
FANCD2		3p25.3	0.2
FANCE		6p21.31	2.6
FANCF		11p14.3	1.7
FANCG	*XRCC9*	9p13.3	9.5
FANCI	*KIAA1794*	15q26.1	1.4
FANCJ	***BRIP1***, *BACH1*	17q23.2	2.0
FANCL	*PHF9, FAAP43*	2p16.1	0.2
FANCM		17q21.2	0.2
FANCN	***PALB2***	16p12.2	0.7
FANCO	***RAD51C***	17q22	0.5
FANCP	***SLX4***	16p13.3	0.5

Table 1. Summary of Fanconi anemia complementation groups. Data parsed from the Rockefeller University Fanconi Anemia Database (http://www.rockefeller.edu/fanconi/); percentages calculated from 1,215 reported FA cases. Bold entries denote accepted gene designation.

which typically develops during the first decade of life (Alter et al., 2003). Additionally, 20% or more of FA patients develop cancer. Though individuals with FA are at increased risk for an array of malignancies, acute myelogenous leukemia is the most common (Kutler et al., 2003). Androgens and hematopoietic growth factors may ameliorate or delay bone marrow failure, though allogeneic stem cell transplantation from a suitable donor is often necessary and remains the only curative treatment for incipient bone marrow failure. Because of inherent hypersensitivity to DNA damaging agents, FA patients require a modified bone marrow transplant procedure, employing low-dose chemotherapy and limited field irradiation (Gluckman et al., 1995; Gluckman and Wagner, 2008). Even in cases where a matched donor is found and engraftment is successful, the patient remains at heightened risk for a number of solid tumors. Readers are referred to a recent review by D'Andrea (D'Andrea, 2010) or Online Mendelian Inheritance in Man (OMIM) entry #227650 for a comprehensive review of the clinical and genetic features of FA.

Hereditary Breast and/or Ovarian Cancer

Inherited mutations in the breast cancer susceptibility genes *BRCA1* and *BRCA2* are responsible for approximately 50% of all hereditary breast cancers and 45% of all hereditary ovarian cancers. Estimates of penetrance of *BRCA1* and *BRCA2* germline mutations vary significantly in different series, but a meta-analysis of 22 population-based studies examining 8,139 index cases, 500 of whom were found to carry a germline mutation in *BRCA1* or *BRCA2* has given valuable average estimates of cancer risk in mutation carriers (Antoniou et al., 2003). The cumulative risk of *BRCA1* mutation carriers developing breast or ovarian cancer by age 70 was found to be 65% (44-78%) and 39% (18-54%), respectively. Inheritance of a single defective *BRCA2* allele was

found to be associated with a 45% (31-56%) and 6% lifetime risk of breast cancer in females and males, respectively, 11% (2.4-19%) lifetime risk of ovarian cancer, and a significantly increased risk of cancers of the prostate, pancreas, larynx, stomach and skin melanocytes (1999; Antoniou et al., 2003). As described previously, individuals with inherited biallelic defects in the *BRCA2* gene develop FA, as *BRCA2* and *FANCD1* are the same gene. Similarly, while germline biallelic defects in the *FANCN/PALB2* and *FANCJ/BRIP1* genes cause FA, inherited monoallelic defects in these same genes account for 2% of non-*BRCA1/2* HBOC families (Rahman et al., 2007; Seal et al., 2006). As in Fanconi anemia, deleterious mutations in the *BRCA1* and *BRCA2* genes are more common in individuals of Ashkenazi Jewish descent. Indeed, approximately 2.5% of Ashkenazi Jews unselected for family history of breast cancer will carry a founder mutation in *BRCA1* (185delAG or 5382insC) or *BRCA2* (6174delT). These three mutations account for 78-96% of all *BRCA1* or *BRCA2* mutations seen in this population (Rubinstein, 2004). A follow-up study from the previously mentioned meta-analysis examined breast and ovarian cancer risks specifically in individuals carrying one of the three Ashkenazi founder mutations and found that generally, cancer risks associated with these three mutations were similar to risks of non-Ashkenazi founder *BRCA1* or *BRCA2* mutations, though the 6174delT *BRCA2* mutation conferred a higher than expected ovarian cancer risk (Antoniou et al., 2005).

The FA/BRCA Pathway

Positional cloning, biochemical analysis and functional complementation studies in families with FA and with HBOC enabled the identification of the genes that now define the 15 complementation groups associated with FA and the two distinct genetic variants of HBOC. One of the hallmarks of FA is cellular sensitivity to DNA crosslinking agents like diepoxybutane

(DEB) or mitomycin C (MMC). Indeed, measurement of chromosomal breaks in primary lymphocytes following exposure to DEB exposure remains a mainstay of FA diagnosis (Auerbach and Wolman, 1976). Because of common clinical findings and hypersensitivity to DEB regardless of complementation group, the products of these genes all presumably operate in a single pathway. It is now known that eight of the 15 FA genes (FANCA, B, C, E, F, G, L and M) assemble a core nuclear ubiquitin ligase complex that regulates the activity of two other FA proteins, FANCD2 and FANCI. Upon recognition of an interstrand crosslink (ICL), FANCM, Fanconi anemia-associated protein 24 (FAAP24) and FANCM-associated histone fold 1/2 (MHF) bind near the ICL and recruit the eight-membered core complex. This assembly then monoubiquitinates FANCD2 and FANCI (Figure 1, Steps 1-2). These modified proteins associate with chromatin and recruit Fanconi anemia-associated nuclease (FAN1), which cooperates with other proteins to excise the ICL, induce a double-strand break (DSB) and prime the broken DNA strands for repair (Figure 1, Steps 2-5). After FAN1-mediated excision to form free 3' flaps of single-strand DNA (ssDNA) (Figure 1, Step 6), the FANCN, FANCJ, BRCA1 and BRCA2 proteins assemble and nucleate the ssDNA with RAD51 (Figure 1, Step 7). This enables invasion of the remaining broken strand and repair by homologous recombination (HR) (Figure 1, Step 8). The products of the FA and BRCA genes are all required for efficient and high-fidelity repair of ICLs.

Not surprisingly, mutation of or deficiency in any of these genes would be expected to abrogate ICL repair and result in hypersensitivity to crosslinking agents. Several traditional cytotoxic agents used in chemotherapy induce ICLs, including cisplatin (and derivatives), nitrosoureas (e.g., carmustine), nitrogen mustards (e.g., cyclophosphamide and melphalan) as well as the

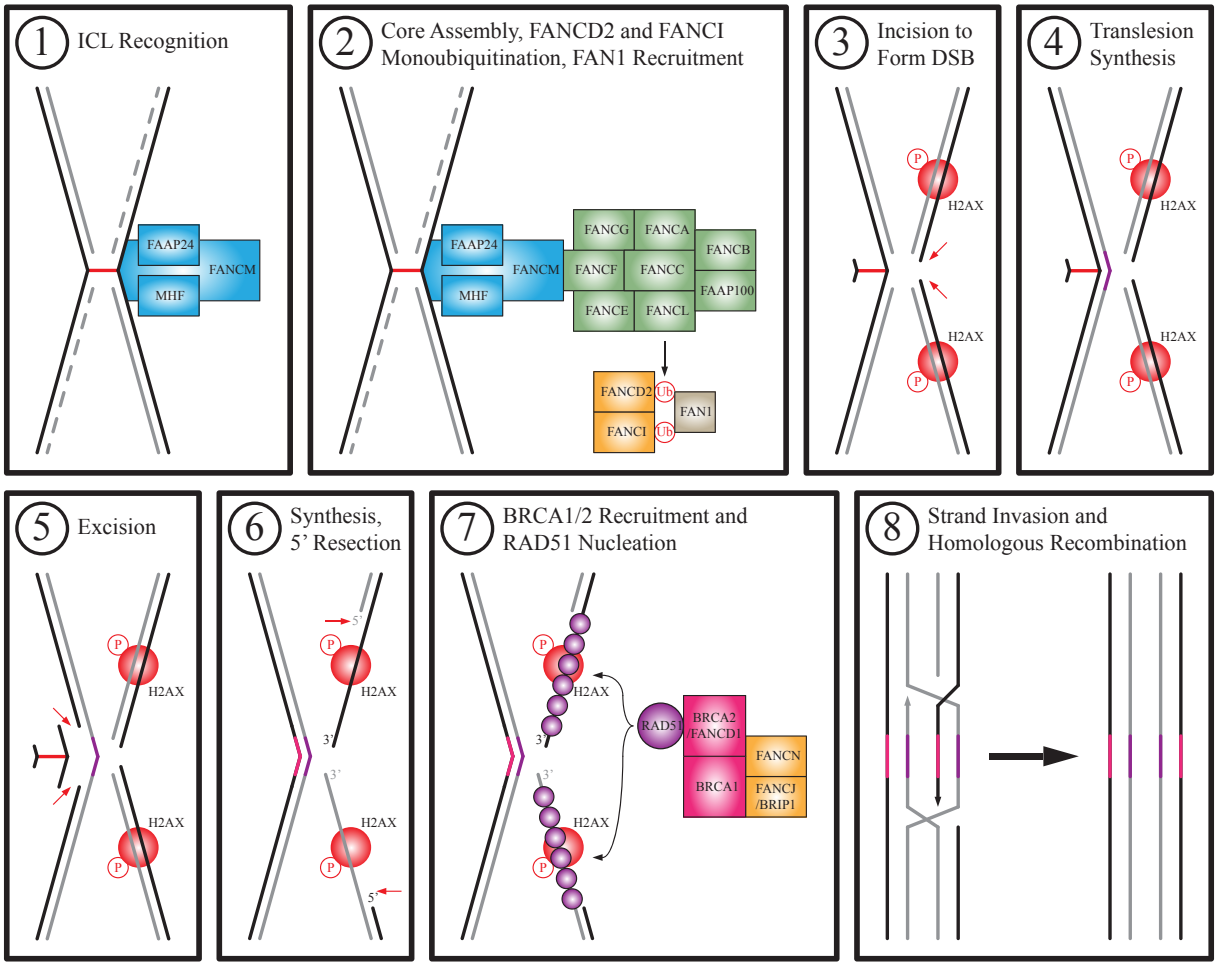
1
ICL Recognition
FAAP24
MHF
FANCM
2
Core Assembly, FANCD2 and FANCI
Monoubiquitination, FAN1 Recruitment
FAAP24
MHF
FANCM
FANCG
FANCA
FANCB
FANCF
FANCC
FAAP100
FANCE
FANCL
FANCD2
FANCI
Ub
FAN1
3
Incision to
Form DSB
P
H2AX
4
Translesion
Synthesis
5
Excision
6
Synthesis,
5' Resection
5'
3'
7
BRCA1/2 Recruitment and
RAD51 Nucleation
RAD51
BRCA2
/FANCD1
FANCN
BRCA1
FANCJ
/BRIP1
8
Strand Invasion and
Homologous Recombination

Figure 1. Schematic of interstrand crosslink recognition and repair by the FA/BRCA pathway.

antitumor antibiotic mitomycin C. Tumors with defects in the FA/BRCA pathway commonly respond more favorably to these agents than do tumors with an intact FA/BRCA pathway. Thus, while inherited mutations in the FA and BRCA genes incite genomic instability and predispose to tumorigenesis, defects in these genes may also be an Achilles' heel. By identifying impairments in the FA/BRCA pathway, it may be possible to tailor cytotoxic chemotherapy to exploit intrinsic vulnerabilities within the DNA repair machinery. Ideally, this approach will lead to better responses to traditional chemotherapeutic regimens and will spare patients the exposure to and side effects of inappropriate chemotherapy. Even more recently, therapeutic strategies based on synthetic lethality have emerged that demonstrate remarkable selectivity for cells with inherited or acquired defects in homologous recombination.

The remainder of this chapter will (1) discuss the relevance of synthetic lethal approaches to tumors with defects in homologous recombination, (2) detail studies which have identified cancer-associated alterations in the FA/BRCA pathway and linked such changes to altered responses to specific chemotherapeutic agents, and (3) review novel approaches to identify, quantitate and manipulate FA/BRCA function in human tumors.

Synthetic Lethality

Targeted anti-neoplastic therapy is the long sought after Holy Grail of cancer medicine. Though we have identified, developed and instituted only a small handful of such agents into oncology, the unprecedented improvements we have observed in diseases like chronic myelogous leukemia have fueled our endeavors to engineer the next generation of miracle drugs. While the bulk of this chapter will discuss genetic and epigenetic variations in DNA repair that induce

hypersensitivity to traditional cytotoxic chemotherapeutic drugs, many breast and most ovarian cancer patients still experience therapeutic failure at some point during the course of their disease, illuminating the need to further elucidate the genetic and evolutionary principles at the interface between cancer cell biology and therapy. Towards this end, a recent advance based on synthetic lethality, a genetic principle first described 90 years ago (Bridges, 1922) shows promise in selectively targeting cancer cells that arise in patients with inherited *BRCA1* and *BRCA2* mutations.

The concept

Synthetic lethality is a genetic state in which simultaneous inactivation of two genes (or pathways) is lethal, while loss of one or the other alone is viable. The therapeutic potential of this principle in oncology was first recognized and demonstrated in cancers arising in *BRCA1* and *BRCA2* mutation carriers. Normal cells in *BRCA1* or *BRCA2* mutation carriers are proficient in DSB repair because one functional allele is sufficient. The breast, ovarian and other malignancies that arise in such individuals follow loss of heterozygosity (LOH) of the remaining normal *BRCA1* or *BRCA2* allele. Thus, only the neoplastic cells that have sustained LOH are defective in DSB repair, and this almost certainly explains the hypersensitivity of *BRCA1/2*-associated breast and ovarian cancers to DNA damaging agents (Cass et al., 2003; Chappuis et al., 2002). While the loss of BRCA1/2 function instigates tumorigenesis in HBOC patients, the unique genetic state present in their cancer cells lends to therapeutic exploitation. Pioneering studies by Ashworth identified that BRCA1 or BRCA2 dysfunction profoundly sensitized cells to inhibitors of poly(ADP)ribose polymerase (PARP), an enzyme involved in base excisional repair (BER) of DNA (Farmer et al., 2005). The underlying reason for this hypersensitivity was

said to be that in the absence of PARP activity, there is persistence of DNA lesions that are normally repaired by homologous recombination. Inhibition of PARP induces synthetic lethality selectively in cells which have sustained LOH at the *BRCA1* or *BRCA2* loci because non-neoplastic (i.e., *BRCA1* or *BRCA2*) heterozygous cells retain HR competence and can thus survive loss of BER capacity (Figure 2). Subsequent studies by Ashworth revealed that deficiency of a number of proteins involved in HR, including RAD51, NBS1, ATR, ATM, CHK1, CHK2, FANCD2, FANCA, and FANCC all induced hypersensitivity to PARP inhibition, implying that the hypersensitivity phenotype is indeed due to failure of HR-mediated repair and that PARP inhibition may not only be a viable therapeutic option for *BRCA1/2*-mutation associated cancers, but rather a wide range of tumors with defects in the HR pathway (McCabe et al., 2006).

PARP inhibitors in BRCA1/2-mutation associated malignancies

Several PARP inhibitors have been developed and are now in clinical evaluation. Among the earliest of these agents was AZD-2281 (olaparib), a potent orally bioavailable PARP inhibitor that was evaluated in a cohort of 60 patients, 22 of whom were carriers of *BRCA1* or *BRCA2* mutations with breast, ovarian or prostate cancers who had previously received multiple treatment regimens. This study reported few adverse effects and 63% of the mutation carriers had objective radiologic or biochemical evidence of tumor response or significant periods of disease stabilization. Importantly, none of the non-mutation carriers demonstrated any objective antitumor activity with AZD-2281 treatment, supporting the notion that PARP and the BRCA1/2 pathway are related in a synthetic lethal manner (Fong et al., 2009). A similar conclusion was reached in another study of 50 ovarian cancer patients, 48 of whom had germline *BRCA1* or

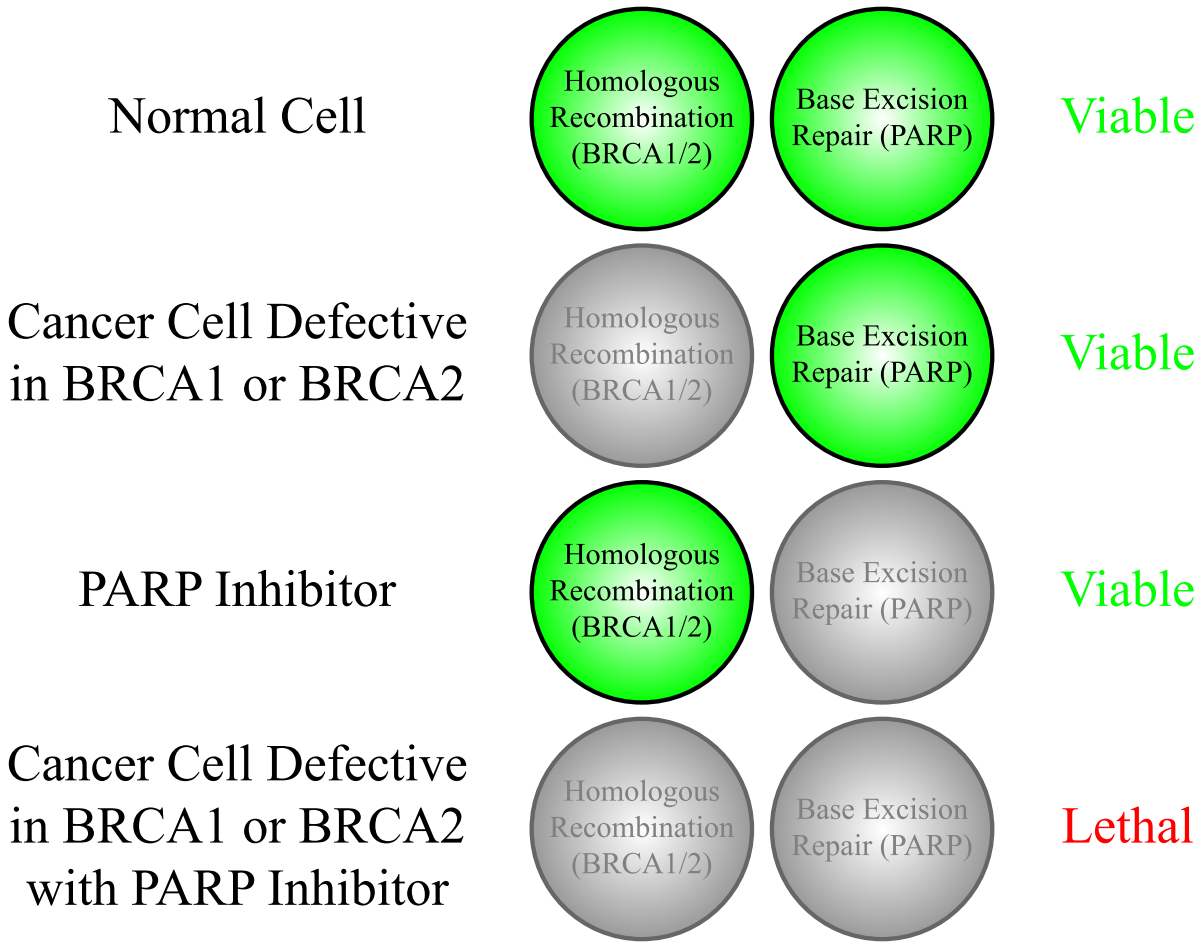
Normal Cell
Homologous Recombination (BRCA1/2)
Base Excision Repair (PARP)
Viable
Cancer Cell Defective in BRCA1 or BRCA2
Homologous Recombination (BRCA1/2)
Base Excision Repair (PARP)
Viable
PARP Inhibitor
Homologous Recombination (BRCA1/2)
Base Excision Repair (PARP)
Viable
Cancer Cell Defective in BRCA1 or BRCA2 with PARP Inhibitor
Homologous Recombination (BRCA1/2)
Base Excision Repair (PARP)
Lethal

Figure 2. Simultaneous inhibition of homologous recombination and base excision repair is synthetic lethal.

BRCA2 mutations, with an overall clinical benefit rate of 46% with olaparib (Fong et al., 2010). Two additional studies enrolling only *BRCA1/2*-mutation-associated advanced breast cancer and recurrent ovarian cancer demonstrated objective response rates of 41% and 33%, respectively, and clinical benefit rates of 52% in both studies for patients on the higher dose of olaparib (Audeh et al., 2010; Tutt et al., 2010). Another recent study by O'Shaughnessy and colleagues evaluated the efficacy of gemcitabine and carboplatin, with or without iniparib, (BSI-201, Sanofi-aventis), another small-molecule proposed to inhibit PARP, in 123 patients with metastatic triple-negative breast cancer. This study did not classify patients based on *BRCA1/2* mutation, but evaluated clinical benefit, overall rate of response, progression-free survival and overall survival. In the intention-to-treat population, the addition of iniparib to chemotherapy increased clinical benefit from 34% to 56%, overall response rate from 32% to 52%, progression free survival from 3.6 to 5.9 months, and overall survival from 7.7 to 12.3 months (O'Shaughnessy et al., 2011). Despite this promising phase 2 trial of iniparib, a phase 3 trial failed to prolong survival in triple-negative breast cancer patients. Two major problems exist in these studies; the first being that the phase 2 trial by O'Shaughnessy (O'Shaughnessy et al., 2011) did not evaluate *BRCA1/2* mutation status or dysfunction, and was thus unable to ascertain whether therapeutic benefit in triple negative lesions was disproportionally skewed towards benefiting *BRCA* mutation carriers. Secondly, the exact mechanism of action for iniparib remains unknown, and a very recent study provides compelling evidence that the drug non-selectively modifies cysteine-containing proteins and is thus not a bona fide PARP inhibitor (Liu et al., 2012). Shortly after the announcement of the phase 3 iniparib failure, AstraZeneca announced that it would not be pursuing phase 3 development of olaparib for *BRCA1/2*-associated breast cancer. The latter decision has caused substantial concern and dismay among

many scientists, physicians and breast cancer patients alike, as many believe there is substantial evidence to support use of olaparib in *BRCA1/2*-associated breast cancer (Guha, 2011). Though the spirited excitement that initially surrounded PARP inhibitors has been replaced by a more cautious enthusiasm, many remain optimistic that this class of agents will find use in breast, ovarian and other malignancies. Above all, the perceived failure of PARP inhibitors in breast cancer testifies to the heterogeneity of this disease. Additional biomarker studies and clinical trials which account for *BRCA* status and other parameters of HR capacity will likely identify subsets of patients who will benefit from these agents.

PARP inhibitors in sporadic malignancies

A study by Hennessy, et. al. evaluated *BRCA1* and *BRCA2* gene integrity and mRNA expression in 235 unselected ovarian cancers and noted that 19% of the tumors in this cohort had a mutation in either *BRCA1* or *BRCA2*. Among the patients with tumor *BRCA1/2* mutations who had provided both tumor and germline DNA for analysis (n = 28), it could be demonstrated that somatic (i.e., acquired) mutation of *BRCA1* and *BRCA2* accounted for 43% and 29% of all mutations identified, respectively. This study estimated that approximately 7% of all sporadic ovarian cancers have sustained a somatic mutation in either *BRCA1* or *BRCA2*. As will be discussed below, this study concurs with others demonstrating that loss of *BRCA1/2* function is associated with improved progression-free survival after platinum-based chemotherapy (Hennessy et al., 2010). Though PARP inhibitor therapy was not used in this study, when considering inherited and somatic mutations in *BRCA1*/*2*, downregulation of BRCA1/2 mRNA or protein, or possessing a *"BRCA*ness" gene expression signature, up to 42% of ovarian cancer patients may benefit from PARP inhibitors (Hennessy et al., 2010; Konstantinopoulos et al.,

2010; Quinn et al., 2007; Swisher et al., 2009; Teodoridis et al., 2005; Weberpals et al., 2009). It is possible that patients with other sporadic malignancies that exhibit defects in the FA/BRCA pathway will enjoy clinical benefit from PARP inhibitors, highlighting the importance of both identifying classes of cancers with such defects, as well as defining diagnostic tools to identify individual patients that will benefit from these therapies.

Though inhibition of base excision repair in cancers with impaired homologous recombination is the first example of synthetic lethality that has been successfully implemented into clinical oncology, malignant cells are richly endowed with unique genetic and epigenetic alterations that may lend to such approaches. Another study by Ashworth and colleagues reported a synthetic lethal relationship between dysfunction of the mismatch repair (MMR) pathway and the dihydrofolatereductase inhibitor methotrexate. Inherited mutations in MMR pathway genes, most commonly *MSH2* and *MLH1*, lead to hereditary non-polyposis colorectal cancer (HNPCC, also known as Lynch syndrome) and dramatically increase risk for ovarian, endometrial, gastric colorectal and other malignancies. Ashworth demonstrated that treatment with methotrexate led to accumulation of oxidized bases in MSH2-deficient cells, but not in cells with an intact MMR pathway (Martin et al., 2009). This finding has led to a phase II trial of methotrexate in MSH2-deficient metastatic colorectal cancer.

Studies which identify additional synthetic lethal relationships between specific recurrent molecular abnormalities in human cancers and "druggable" targets are sorely needed and would offer great promise in clinical oncology.

FA/BRCA Dysfunction in Human Cancers and Association with Therapeutic Sensitivity

Though Fanconi anemia is a rare recessive disorder and hereditary breast and/or ovarian cancer accounts for a minority of all breast and ovarian malignancies, polymorphisms with functional significance, somatic mutations, and epigenetic silencing of the BRCA and/or FA genes are relatively common in diverse human cancers. Numerous studies have correlated these genetic and epigenetic signatures with clinicopathological features of malignancy, response to chemotherapeutic agents, and with prognosis and survival (Table 2).

Breast Cancer

BRCA1, BRCA2 and the Intrinsic Molecular Subtypes of Human Breast Cancer

Breast cancer is a heterogeneous disease at both the histological and molecular level. Gene expression profiling of large cohorts of human breast cancers has established five major intrinsic subtypes of invasive breast cancer: luminal type A, luminal type B, basal-like, $HER2^{+}$, and normal breast-like (Sorlie et al., 2001). Retrospective analysis of patient outcomes in these studies demonstrated that specific molecular taxonomies are strongly correlated with unfavorable clinical behavior and poor overall survival. Specifically, the basal-like and HER2-overexpressing tumors are associated with an aggressive clinical course, resistance to chemotherapy, and increased risk of distant metastasis (Rodriguez-Pinilla et al., 2006; Sorlie et al., 2001; Sorlie et al., 2003). The basal-like breast cancers (BLBCs) were so named because these neoplasms consistently express molecules normally confined to the basal/myoepithelial compartment of the ductal and lobular epithelium. BLBCs account for approximately 15% of all invasive breast cancers and are typically of high histological grade, demonstrate high mitotic indices, mutations in the *TP53* tumor suppressor gene, and almost uniformly lack expression of

		Loss of Gene Function		
FA/BRCA Gene	**Disease**	**Increased Sensitivity or Survival**	**Decreased Sensitivity or Survival**	**Other References**
BRCA1	Hereditary Breast Cancer***	Chappuis (2002)	Ansquer (1998) Chappuis (2000) Stoppa-Lyonnet 2000)	Bordeleau (2010) Chappuis (2011) Gaffney (1998) Johannson (1998) Robson (1998) Verhoog (1998)
	Sporadic Breast Cancer	Asakawa (2010)		Thompson (1995) Wei (2008)
	Hereditary Ovarian Cancer***	Cass (2003) Gallagher (2011) Lacour (2011) Norquist (2011) Radosa (2011) Rubin (1996) Swisher (2008) Tan (2008) Vencken (2011)		Butler (2002) Johannson (1998)
	Sporadic Ovarian Cancer	Konstantinopoulos (2010)† Quinn (2007) Swisher (2009) Taniguchi (2003) Teodoridis (2005) Weberpals (2009)	Chiang (2006)	
FANCC	Early-Onset Breast Cancer		Sinha (2008)	
	Late-Onset Breast Cancer		Sinha (2008)	
FANCD1 / BRCA2	Hereditary Breast Cancer***	Chappuis (2002)	Chappuis (2000)	Bordeleau (2010) Gaffney (1998) Robson (1998)
	Hereditary Ovarian Cancer***	Cass (2003) Gallagher (2011) Lacour (2011) Norquist (2011) Sakai (2009) Tan (2008) Vencken (2011)		
	Sporadic Ovarian Cancer	Konstantinopoulos (2010)†		
FANCD2	Hereditary Breast Cancer*			van der Groep (2008)
	Sporadic Breast Cancer	van der Groep (2008)		
FANCF	Sporadic Breast Cancer			Tokunaga (2009) Wei (2008)
	Sporadic Ovarian Cancer	Taniguchi (2003)	Lim (2008)	Swisher (2009) Teodoridis (2005) Wang (2006)
	Sporadic Ovarian Germ Cell Tumor			Dhillon (2004)
FANCN / PALB2	Hereditary Breast Cancer**			Potapova (2008)
	Sporadic Breast Cancer			Potapova (2008)
	Hereditary Ovarian Cancer***			Potapova (2008)
	Sporadic Ovarian Cancer			Potapova (2008)

* Associated with *BRCA1*
** Associated with *BRCA2*
*** Associated with *BRCA1* or *BRCA2*
† Based on a non-FA/BRCA Signature

Table 2. Summary of clinical studies evaluating FA/BRCA dysfunction and association with sensitivity to DNA-damaging agents.

estrogen receptor (ER), progesterone receptor (PgR), and HER2 (Turner and Reis-Filho, 2006; Turner et al., 2007). Due to the absence of these receptors, BLBCs are not amenable to the targeted anti-estrogen and anti-HER2 therapies that have drastically improved survival of patients diagnosed with luminal-type or HER2-overexpressing tumors. Traditional cytotoxic chemotherapy is the only approved available systemic treatment for BLBCs. Accordingly, among all molecular classifications of breast cancer, the basal-like malignancies are associated with the most aggressive clinical behavior and poorest prognosis (Sorlie et al., 2001).

An intimate correlation has been recognized between the basal-like phenotype and deficiency of BRCA1. Transcriptional profiling of large cohorts of *BRCA1*-mutated breast cancers has revealed that these tumors generally cluster in the basal-like subtype (Foulkes et al., 2003; Lakhani et al., 2005; Sorlie et al., 2003). Moreover, immunohistochemistry studies reveal that nearly 70% of *BRCA1*-mutated breast cancers express basal cytokeratins and lack expression of ER, whereas this immunophenotype is present in less than 9% of matched control tumors (Lakhani et al., 2005). Sporadic malignancies in which BRCA1 has been transcriptionally or epigentically silenced also have a strong tendency to be of the basal-like phenotype (Thompson et al., 1995; Turner et al., 2007). Conversely, tumors which maintain expression of functional BRCA1 are almost uniformly luminal type cancers and are accordingly associated with more indolent clinical courses, responsiveness to endocrine therapies, and improved survival (Catteau et al., 1999; Yang et al., 2001). Taken together, these findings suggest that loss of BRCA1 expression and/or function has a causal role in the development of the basal-like phenotype. Though this association is now well-supported, the molecular consequences of BRCA1 deficiency that result in the generation of BLBCs remain undefined.

Recent studies support a model in which BRCA1 is necessary for the normal luminal differentiation program within the mammary gland. *In vitro* and *in vivo* studies have revealed that loss of BRCA1 expression in mammary epithelial cells leads to marked dysplasia and failure of terminal luminal epithelial cell differentiation (Furuta et al., 2005; Lim et al., 2009; Liu et al., 2008). These morphological abnormalities are associated with exaggerated expression of basal/myoepithelial antigens and enrichment of cells with expression of the putative stem/progenitor cell marker ALDH1A1 (Lim et al., 2009; Liu et al., 2008; Xu et al., 1999). While these findings would suggest that BRCA1 deficiency may cause BLBCs by causing expansion of the basal/myoepithelial population, a recent study has documented profound expansion of the $CD49f^{+}/EpCAM^{+}$ luminal progenitor population in the pre-malignant breast tissue of *BRCA1*-mutation carriers. The same study revealed that the luminal progenitor population, and not the basal/myoepithelial population, more closely resembles the transcriptional profile of BLBCs (Lim et al., 2009). Since BRCA1 deficiency is known to result in genetic instability, cell cycle abnormalities, and aberrant centrosome duplication (Deng, 2006), it is plausible to assert that BRCA1 deficiency results in stalled terminal differentiation of luminal progenitor cells that are genetically unstable and are destined to produce aggressive basal-like malignancies.

Inherited mutations in *BRCA1* or *BRCA2/FANCD1* account for only 5-10% of all breast cancers. The remaining 90-95% are sporadic in nature, and demonstrate tremendous molecular and morphological diversity. Immunohistochemically, breast cancers are classified into three major groups depending on expression of ER, PR and HER2. Approximately 60% of all breast cancers

express ER and/or PR and are amenable, to varying degrees, to endocrine therapies that are aimed at regulating estrogen production or signaling through the estrogen receptor. The second class, accounting for roughly 25% of all breast cancers, are those that have amplified HER2 expression, but generally lack ER and PR expression. These lesions are more aggressive than those without HER2, but agents like trastuzumab (Herceptin®) and lapatinib (Tykerb®) have dramatically improved clinical outcomes in HER2+ breast cancer. The third class, the so called "triple-negative" (TN) malignancies lack expression of ER, PR and HER2. These tumors are sensitive to neither endocrine therapies nor HER2-targeted therapies and exhibit aggressive biological features. Approximately 15% of all invasive breast cancers are of the TN subtype, though these tumors are overrepresented in patients with metastatic disease and account for a discordantly high proportion of breast cancer-related deaths. The aggressive biological properties of TN breast cancers coupled with a paucity of approved targeted therapeutic agents makes TN breast cancer an enduring clinical obstacle. Fortunately, and of direct relevance to this dissertation, TN cancers of both sporadic and hereditary (i.e., *BRCA1/2*-related) origin appear to have recurrent defects in double-strand break (DSB) repair, due largely to genetic or epigenetic perturbations of the *BRCA1* gene (Turner et al., 2007). Chemotherapeutic approaches aimed at exploiting such defects are being intensely evaluated and may dramatically improve clinical outcomes for TN breast cancer patients.

Because of the unique natural history of *BRCA1/2*-associated breast cancer, several groups sought to assess whether recurrence and/or survival differed between *BRCA*-associated and sporadic cancers. Many of these early studies reported conflicting data, with several noting no significant difference in clinical course (Gaffney et al., 1998; Johannsson et al., 1998; Robson et

al., 1998; Verhoog et al., 1998), and others reporting adverse outcomes in *BRCA1 or BRCA2* mutation carriers (Ansquer et al., 1998; Chappuis et al., 2000; Stoppa-Lyonnet et al., 2000). These studies potentially conflict with another early study by Chappuis, et. al., who demonstrated a more favorable objective response to DNA damaging chemotherapy in *BRCA1/2*-associated breast cancers than in sporadic tumors, in which 91% of breast cancers in *BRCA1/2* mutation carriers demonstrated clinical complete response (cCR) and 44% pathological complete response (pCR) compared to only a 30% cCR and 4% pCR in non-carriers after three or four cycles of anthracycline-based neoadjuvant chemotherapy (Chappuis et al., 2002). The higher response rate observed in carriers was presumed to be due to impaired DNA repair mechanisms and hypersensitivity to chemotherapy in those cancers with BRCA pathway defects. This group was one that had immediately prior to this study demonstrated that *BRCA1/2*-associated breast cancer was associated with a worse prognosis (Chappuis et al., 2000). Using data from yet another study of 292 Ashkenazi Jewish breast cancer patients, they offered an explanation for this apparent paradox, as it was observed that overall survival in *BRCA1*-associated cancers was poorer only in women who did not receive adjuvant chemotherapy, positing that the less favorable outcome observed in some retrospective studies may be explained, at least in part, by the omission of chemotherapy in these historical series (Chappuis et al., 2011). Bordeleau et al., recently published a highly informative critical analysis of early and contemporary studies addressing the prognosis of *BRCA1*/2-associated breast cancers. In addition to addressing methodological and statistical limitations of many of these studies, the major conclusion of this analysis is that prognosis of *BRCA*-associated breast cancer appears to be similar to that observed in sporadic breast cancer (Bordeleau et al., 2010). It is important to note that in many of these studies, *BRCA1*- and *BRCA2*-associated breast cancers were analyzed

together, despite the obvious histological and molecular differences between these groups. The *BRCA2*-associated breast cancers appear in most respects to resemble the more common hormone receptor-positive cancers that comprise the bulk of sporadic lesions, while the pathogenesis of *BRCA1*-associated tumors is quite distinct and, as noted previously, leads in most cases to triple-negative breast cancer. It is possible that alternate trends may have emerged in some of these studies had the *BRCA1*- and *BRCA2*-associated tumors been treated as biologically distinct entities instead of a unified group of familial cancers.

Given the heterogeneous nature of sporadic breast cancer, many recent studies have sought to directly examine the integrity and/or functionality of various components of the FA/BRCA pathway as potential biomarkers of therapeutic efficacy. To study the DNA damage response immediately following neoadjuvant chemotherapy, Asakawa and colleagues collected core needle biopsies before and 18-24 hours after the first cycle of epirubicin plus cyclophosphamide (EC) and examined cellular localization of DNA damage repair proteins, including BRCA1, γH2AX and RAD51 (Asakawa et al., 2010). This study noted that tumors with low baseline staining for BRCA1 nuclear foci exhibited a greater reduction in tumor volume than those with high BRCA1 staining following EC therapy. Moreover, this study devised a simple DNA damage response (DDR) scoring system which took into account baseline (i.e., pre-therapy) γH2AX, BRCA1 and RAD51 foci formation as well as post-EC RAD51 staining and noted that DDR score was inversely correlated with tumor volume reduction after EC, and was more informative than either nodal status or primary tumor size in predicting response (Asakawa et al., 2010). Unlike other tumor suppressors involved in the pathogenesis of breast cancer, somatic mutational inactivation of *BRCA1* is an exceedingly rare event. Our group was the first to

demonstrate that downregulation of BRCA1 was a relatively common event in sporadic breast cancer progression (Thompson et al., 1995). Many studies have since identified non-mutational means of BRCA1 silencing or dysfunction in sporadic breast cancer, including methylation of the *BRCA1* promoter and upregulation of microRNAs (miRNAs) that target the BRCA1 transcript (Garcia et al., 2011; Moskwa et al., 2011). Wei et. al. examined methylation of the *ESR1* (estrogen receptor α), *BRCA1* and *FANCF* promoters in a cohort of 120 sporadic primary breast cancers and noted that *BRCA1* promoter methylation was present in 20% of their cohort. Methylation of *BRCA1* was significantly correlated with methylation of *ESR1*, in agreement with findings that tumors with low or absent expression of BRCA1 are very commonly ER-negative (Wei et al., 2008b). This same study only identified one out of the 120 breast cancers which exhibited *FANCF* promoter methylation. Similarly, another study of Japanese primary breast cancers reported only four out of 99 primary tumors exhibiting *FANCF* promoter methylation (Tokunaga et al., 2009). The *FANCN*/*PALB2* promoter was identified to be methylated in only four of 60 sporadic breast cancers and 2 of 8 *BRCA2*-associated breast cancers (Potapova et al., 2008). Unlike methylation of *FANCF* and *FANCN/PALB2*, molecular changes affecting FANCC and FANCD2 expression are common in sporadic and hereditary breast cancer. Sinha and colleagues collected 106 primary breast cancers and segregated them into early- and late-onset cohorts (≤ 40 years of age and > 40 years of age, respectively) and examined deletions and promoter methylation in the 9q22.32-22.33 chromosomal region, which contains the *FANCC* gene and is altered in a wide variety of tumors. Deletion or promoter methylation of the *FANCC* gene was observed in 53% of early-onset and 59% of late-onset tumors, suggesting that genetic and epigenetic alterations affecting *FANCC* expression are very common in both early- and late-onset sporadic breast cancers. Either form of alteration in *FANCC* was significantly associated

with deletion of *BRCA2* in both early- and late-onset tumors but only with deletion of *BRCA1* in early-onset tumors. In both cohorts, patients with alterations in *FANCC* had poorer overall survival than those with intact and unmethylated *FANCC* genes (Sinha et al., 2008). Conversely, van der Groep et. al. examined FANCD2 expression by immunohistochemistry in two cohorts of sporadic breast cancers and 25 *BRCA1*-associated breast cancers and correlated FANCD2 immunopositivity with several clinicopathological features and prognosis (van der Groep et al., 2008). FANCD2 staining was positively correlated with Ki-67, cyclin A, mitotic activity index (MAI), and p53 staining, but negatively correlated with age, ER, and PR positivity. The association between FANCD2 and proliferative markers was suggested to be a reflection of the biological function of FANCD2 in maintaining integrity of the genome during DNA replication. In a second cohort of 122 sporadic breast cancer cases with annotated survival data, high FANCD2 expression was associated with an unfavorable prognosis, and when analyzed with tumor size, lymph node status, ER and grade, FANCD2 appeared to have significant independent prognostic value for overall survival (van der Groep et al., 2008). Interestingly, in the original cohort of 96 specimens, 19% and 10% of the sporadic and *BRCA1*-associated cases, respectively, stained completely negative for FANCD2. It is likely that a genetic or epigenetic hit at the *FANCD2* locus is responsible for loss of FANCD2 expression. The lower incidence of FANCD2 negativity in the *BRCA1*-associated cohort may be explained by reasoning that a second hit (i.e., in *FANCD2*) would be less advantageous for a cancer cell with a pre-existing hit in the same pathway (i.e., in *BRCA1*) than for one in which the entire FA/BRCA pathway is intact. The authors in this study speculate that the link between high FANCD2 expression and poor prognosis may be a reflection of the high proliferative potential of these tumors, though it is

equally interesting to speculate that high FANCD2 expression confers intrinsic resistance to chemotherapy by way of increasing repair capacity (van der Groep et al., 2008).

Ovarian Cancer

As is the case with breast cancer, only 10-15% of all epithelial ovarian cancers (EOC) are hereditary in nature. Unlike breast cancer, where first-line chemotherapy is often anthracycline-based, EOCs have been treated with platinum-based regimens for several decades. While anthracyclines are known to induce DSBs through inhibition of the re-ligation step of topoisomerase II (Tewey et al., 1984), repair of these lesions does not necessarily require the FA/BRCA pathway, as most DSBs in higher eukaryotes are repaired via non-homologous end joining (NHEJ) (Shrivastav et al., 2008). Of note, anthracyclines can also form formaldehyde-mediated covalent DNA adducts, and *in vitro* studies have suggested that HR, rather than NHEJ, plays a critical role in resolving this minor form of damage (Spencer et al., 2008). Platinum-based agents induce multiple forms of DNA damage as well, with approximately 96% of the damage being intrastrand crosslinks between 5'-GG-3', 5'-AG-3' and 5'-GNG-3' nucleotides. The remaining 4% of the damage is interstrand crosslinks between antiparallel 5'-GC-3' nucleotides on opposing strands (Eastman, 1986) (Figure 3). Intrastrand crosslinks are repaired primarily by nucleotide excision repair (NER), while the interstrand crosslinks, though drastically less numerous, are more biologically toxic and require the FA/BRCA pathway to be repaired (Deans and West, 2011). Because of the longstanding application of platinum-based agents in EOC, the necessity of the FA/BRCA pathway in repairing interstrand crosslinks, and the association of *BRCA1/2* with hereditary ovarian cancer, this group of diseases has been much

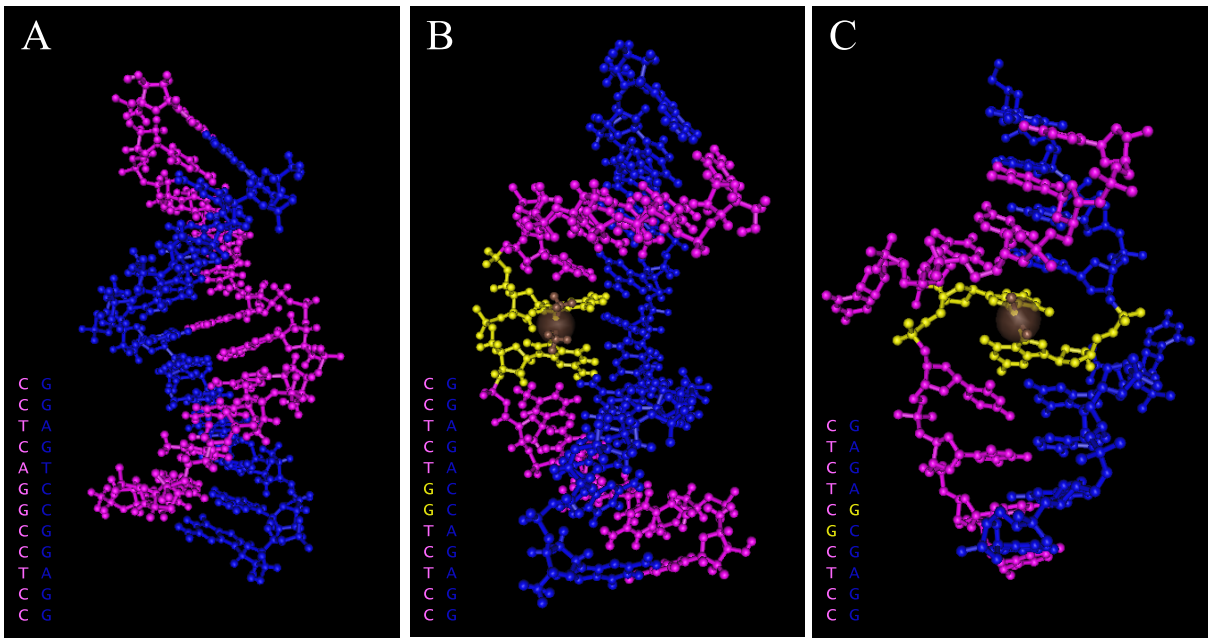
A
B
C

Figure 3. Platnim-induced structural perturbations of DNA. (A) Normal DNA; structure derived from MMDB ID 53875 (Wu, 2007). (B) A cisplatin-induced GpG intrastrand crosslink; structure derived from MMDB ID 47796 (Gelasco, 1998). (C) A cisplatin-induced inter-strand crosslink; structure derived from MMDB ID 47723 (Coste, 1999). Crosslinked nucleotides indicated in yellow. All structures rendered in Cn3D (National Center for Biotechnology Information).

more robustly studied with respect to FA/BRCA defects and chemosensitivity in both sporadic and hereditary malignancies.

Similar to the early study on *BRCA1/2*-associated breast cancers (Chappuis et al., 2002), several early reports demonstrated that *BRCA1*- or *BRCA2*-associated ovarian cancers may be associated with a more favorable clinical course. A study by Rubin et. al. of 53 advanced-stage EOC patients with a germline *BRCA1* mutation noted median survival of 77 months, compared to 29 months in non-*BRCA1*-associated advanced EOC patients (Rubin et al., 1996). Similar results were obtained in a later study which reported that EOC patients heterozygous for either *BRCA1* or *BRCA2* had higher response rates to primary therapy compared to sporadic cancers and that *BRCA1/2* mutation-associated EOC patients had longer disease free survival (49 vs. 19 months) and overall survival (91 months vs. 54 months) than non-*BRCA* associated EOC (Cass et al., 2003). In contrast, a study examining *BRCA1*-associated breast and ovarian cancer reported an initial survival advantage in *BRCA1*-assocaited EOC that disappeared with time, ultimately suggesting a similar or potentially worse prognosis than sporadic EOC (Johannsson et al., 1998). These results are in concert with a study examining EOCs with *BRCA1* dysfunction, defined as either documented mutation (germline or somatic) or *BRCA1* promoter methylation leading to low or absent BRCA1 mRNA expression, in which no significant survival differences were noted between tumors based on BRCA1 functionality (Buller et al., 2002). Despite these conflicting early reports, most contemporary studies have documented improved survival in EOC patients with *BRCA1* or *BRCA2* mutations compared to sporadic EOC (Gallagher et al., 2011; Lacour et al., 2011; Radosa et al., 2011; Tan et al., 2008; Vencken et al., 2011; Yang et al., 2011).

Regarding sporadic EOCs and dysfunction of the FA/BRCA pathway, Taniguchi and colleagues proposed a model based on *in vitro* studies whereby platinum sensitive ovarian cancer cells have methylated the *FANCF* promoter, thus impairing monoubiquitination of FANCD2 (Figure 1, Step 2) and efficient interstrand crosslink repair. This same study generated a cisplatin-resistant clone of a hypersensitive EOC cell line and demonstrated that the region immediately upstream of the *FANCF* transcriptional start site was almost globally unmethylated in the resistant cell line (Taniguchi et al., 2003). In a cohort of 19 women with surgically resected primary ovarian tumors, this group identified methylation of *FANCF* in up to 21% of clinical specimens (Taniguchi et al., 2003). This is comparable to studies by Wang and Lim demonstrating methylation of *FANCF* in 28% and 13% of primary ovarian cancers, respectively (Lim et al., 2008; Wang et al., 2006). The Wang study also noted that 67% of all specimens examined exhibited decreased FANCF protein expression (Wang et al., 2006). Though inconsistent with *in vitro* data, the study by Lim et. al. noted that tumors with unmethylated *FANCF* exhibited a more favorable progression-free survival than those with methylated *FANCF*, though no association was seen with overall survival (Lim et al., 2008). Another study by Teodoridis, et. al. aimed to examine methylation of *FANCF*, *BRCA1*, and other candidate DNA repair genes in clinical EOC specimens. Contrary to the studies by Taniguchi, Wang and Lim (Lim et al., 2008; Taniguchi et al., 2003; Wang et al., 2006), this group identified that none of the 106 stage III/IV EOCs in their cohort demonstrated *FANCF* promoter methylation, though *BRCA1* promoter methylation was observed in 8.8% and 23.1% of the stage III and IV tumors, respectively (Teodoridis et al., 2005). Similarly, another study of 93 sporadic epithelial ovarian cancers only noted *FANCF* methylation in 3% of their specimens (Swisher et al., 2009). The discrepancy between these studies with respect to *FANCF* methylation may be due to methodological differences, as well as

the fact that several studies may have included some germ cell tumors and not strictly EOCs. This is perhaps supported by another study conducted by Dhillon and colleagues which showed that 24% of ovarian germ cell tumors exhibited *FANCF* promoter methylation (Dhillon et al., 2004). In the Teodoridis study, tumors with *BRCA1* methylation failed to fall into a cluster characterized by methylation of other genes, suggesting that *BRCA1* methylation may occur by a distinct process or under a different selective pressure. Methylation of at least one candidate gene involved in DNA repair (*BRCA1*, *GSTP1* or *MGMT*) was significantly associated with response to chemotherapy, as 100% of patients with at least one of these three genes methylated responded to therapy. When evaluating *BRCA1* methylation independently of *GSTP1* and *MGMT*, the difference was less robust, but still significant (Teodoridis et al., 2005). In contrast, a comparatively small study examining survival with respect to *BRCA1* promoter methylation found that sporadic tumors with *BRCA1* methylation carried poorer prognosis than *BRCA1*-mutation associated cancers or sporadic cancers with an unmethylated *BRCA1* promoter (Chiang et al., 2006). Though the results from this study are interesting, more numerous studies, including those with larger cohorts have since suggested that low BRCA1 expression is associated with prolonged survival. One such study of 51 sporadic ovarian cancer patients receiving platinum-based chemotherapy reported median observed survival of 46 versus 33 months for patients with lower and higher BRCA1 mRNA expression, respectively (Weberpals et al., 2009). High BRCA1 mRNA expression appeared to be especially unfavorable in individuals with residual disease after surgical cytoreduction. This pattern also emerged in a study by Quinn and colleagues who compared BRCA1 mRNA expression and survival in sporadic EOC and found that patients undergoing platinum-based chemotherapy with low or intermediate expression of BRCA1 mRNA had a mean 57.2 month overall survival compared to

18.2 months in those with high levels of BRCA1 mRNA expression (Quinn et al., 2007). This study also found that BRCA1 mRNA expression, while inversely correlated to platinum sensitivity, was positively correlated with sensitivity to taxane-containing regimens. BRCA1 is known to be required for G2/M arrest after microtubule poisons, and it has been shown *in vitro* that tumors with high expression of BRCA1 are sensitive to these agents, while deficiency of BRCA1 leads to relative resistance (Lafarge et al., 2001; Mullan et al., 2001; Quinn et al., 2003; Tassone et al., 2005). Accordingly, in the poor prognosis cohort with high BRCA1 mRNA expression, addition of a taxane produced a trend for higher survival (23.0 versus 18.2 months) (Quinn et al., 2007). Perhaps most convincing, a study of 115 sporadic ovarian cancers, including 31 which had paired pre- and post-chemotherapy samples, found that 34% and 42% of primary EOCs had low BRCA1 and BRCA2 protein expression, respectively (Swisher et al., 2009). Methylation of *BRCA1* was only observed in 7% of this cohort, implying that loss of BRCA1 occurs most commonly by means other than promoter methylation. As was observed in previous studies, loss of BRCA1 protein in primary neoplasms was associated with significantly better overall survival (62 months versus 45 months) than for tumors which retained BRCA1 expression (Swisher et al., 2009), though there was no association between BRCA1 expression and likelihood of complete response to initial chemotherapy as was seen in a previous study (Taniguchi et al., 2003). Moreover, for paired specimens in which the primary tumor exhibited low or intermediate BRCA1 or BRCA2, expression of these proteins was increased in 62% and 71% of the recurrent carcinomas, respectively (Swisher et al., 2009). As in sporadic breast cancer, methylation of the *FANCN/PALB2* promoter in sporadic EOC appears to be relatively uncommon, with only four of 53 primary tumors exhibiting this epigenetic alteration in one study (Potapova et al., 2008). Lastly, a recent clinical study by Konstantinopoulos and

colleagues established a "*BRCA*ness" signature by interrogating gene expression data from 61 EOC patients, 34 of whom were known to have *BRCA1/2* mutations. The classifier that emerged from this analysis was based on expression of sixty genes, of which notably, none are known to play significant roles in the FA/BRCA pathway. When applied to another cohort of seventy patients with sporadic EOC, patients with the "*BRCA*ness" profile had improved disease free and overall survival (34 versus 15 months and 72 versus 41 months, respectively) compared to patients whose tumors did not exhibit this signature (Konstantinopoulos et al., 2010). Though the concept of "*BRCA*ness" and differential sensitivity in familial and sporadic EOCs has been appreciated in both breast and ovarian cancer for over a decade, most of the prior methodologies used to define "*BRCA*ness" relied on an assumption that the defect was mechanistically associated with HR (e.g., *BRCA1/2* promoter methylation). Because EOCs may evolve defects in HR by diverse mechanisms, this last study is notable in that it uses an approach which omits mechanistic assumptions and thus is poised to identify patients whose tumors exhibit defects in the HR pathway due to varied genetic or epigenetic perturbations.

Assessing FA/BRCA Pathway Function

In virtually all of the studies discussed, assessment of FA/BRCA function was made either retrospectively or incidentally during therapy, having been neither a prescribed diagnostic criterion nor having any bearing on clinical management of the patient. It is nonetheless evident that dysfunction of the FA/BRCA pathway is relatively common in several prevalent cancers, and that, in select clinical scenarios, *a priori* knowledge of repair capacity may allow selection of more appropriate therapeutic agents and enable personalized cytotoxic chemotherapy, with the obvious advantage of improving clinical outcomes. In order to make assessment of FA/BRCA

pathway integrity and function a reality, we must develop rapid assays that are informative of pathway function as a whole, do not require specialized instrumentation, and are both reproducible and relatively economical. Several methodologies, including many that have been discussed, may be performed on preserved tissue or fluid specimens, while others will require an *ex vivo* genotoxic insult in order to measure the ability of cells to mount and/or execute an effective repair response. Though implementation of such assays into clinical practice is not currently a reality, several studies have made promising strides in identifying potentially useful reporters of FA/BRCA pathway function.

Following induction of a double-strand break (either directly by ionizing radiation or as part of the repair process of interstrand crosslinks), components of the FA/BRCA pathway will assemble into punctate nuclear foci (Figure 4). Within minutes of sustaining a DSB, phosphorylated histone H2AX (serine 139) can be detected (Rogakou et al., 1998). This is one of the earliest recognized markers that damage has been sustained, and this modified histone (designated γH2AX) serves as a docking site for components of the FA/BRCA pathway to initiate homology-directed repair (Figure 1). Following successful resolution of the DSB, H2AX becomes dephosphorylated. A recent study by Olive and Banáth examined the kinetics of γH2AX foci formation and response to cisplatin in several human and rodent cell lines with differential capacity to repair DNA damage. They found that initial rates of H2AX phosphorylation were unrelated to drug sensitivity or proficiency in crosslink repair. Conversely, the fraction of cells that retained γH2AX foci at 24 hours after drug treatment (i.e., were unable to resolve damage) also had marked loss of clonogenic growth potential (Banath et al., 2004; Olive and Banath, 2009). Thus, persistence of γH2AX foci formation at delayed

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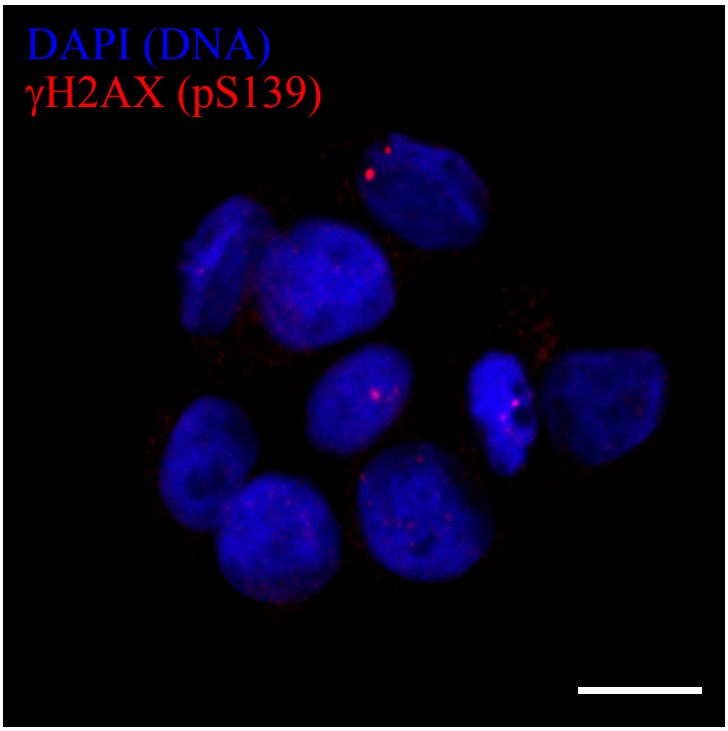


+10 Gy Ionizing Radiation

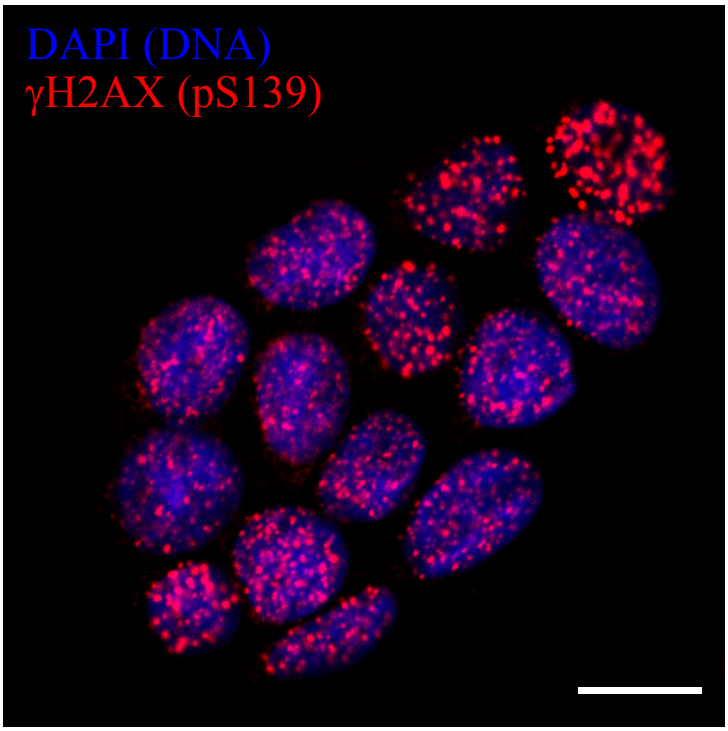


Figure 4. Damage-induced foci. MCF7 breast adenocarcinoma cells were grown in chamber slides and exposed to zero or 10 Gy ionizing radiation from a ^{137}Cs source (5.46 $Gy \cdot min^{-1}$). Cells were fixed and immunostained for γH2AX (pS139) at two hours post irradiation. DNA was counterstained with DAPI.

timepoints may be a sensitive marker of defective DNA repair and could represent a clinically feasible assay for identifying tumors which may be hypersensitive to DNA crosslinking agents or IR.

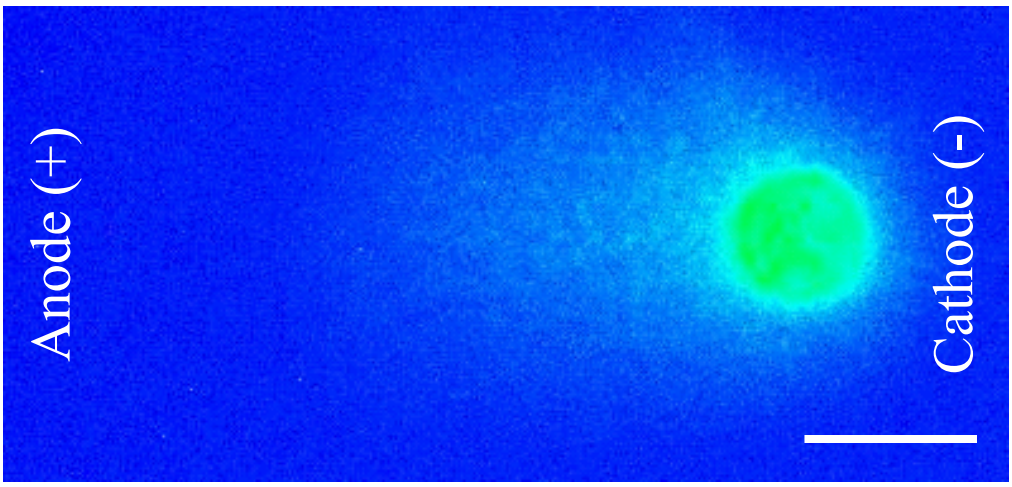
Because of the large number of proteins that form the dynamic FA/BRCA complex, one could argue that examining the function of upstream components (i.e., FANCM or even γH2AX) could be less informative of overall pathway integrity than assessing function of critical downstream components. Several studies have examined FANCD2 and RAD51 subnuclear localization with respect to chemosensitivity *in vitro* and *in vivo*. A study by Willers and colleagues examined biomarkers related to FANCD2 function in virally immortalized fibroblasts from a patient with complementation group D2 Fanconi anemia and demonstrated that retroviral complementation of FANCD2 restored subnuclear FANCD2 assembly in response to DNA damage and conferred resistance to both MMC and H_2O_2 (Willers et al., 2008). This same group soon published a report demonstrating the feasibility of assessing BRCA1, FANCD2 and RAD51 foci on fresh breast cancer biopsy specimens following *ex vivo* irradiation. They examined seven locally advanced sporadic breast cancers for these biomarkers and reported that four of the seven exhibited a defective foci response (Willers et al., 2009). Notably, three of the four tumors with defects were of the triple-negative phenotype, which as previously mentioned has been associated with BRCA1 deficiency (Turner and Reis-Filho, 2006; Turner et al., 2007). After this small pilot study, Graeser et. al. examined RAD51 foci formation in 68 sporadic breast cancer patients who received neoadjuvant anthracycline-based chemotherapy. In their study, patients underwent a core biopsy 24 hours after the first cycle of chemotherapy and this specimen was assessed for RAD51 subnuclear assembly by immunofluorescence microscopy. Decreased

RAD51 focus formation was present in 26% of their specimens and correlated with high histologic grade, high baseline proliferation, and the triple-negative phenotype. Importantly, a low RAD51 score was strongly predictive of response to therapy, with 33% of patients with low RAD51 achieving pCR compared with only 3% of patients with an intact RAD51 response (Graeser et al., 2010). While somewhat invasive, this strategy has precedence in other tumors (e.g., osteosarcoma) and may be tremendously useful in predicting response and selecting appropriate chemotherapeutic agents. Moreover, this type of analysis may be less technically challenging and more widely applicable for tumors in which neoplastic cells are more readily available for examination following chemotherapy (e.g., leukemic cells from blood and ovarian/peritoneal cancer cells from ascitic fluid in patients receiving intraperitoneal chemotherapy).

An alternate means of assessing repair capacity is to examine not a molecular marker associated with repair, but the physical repair of DNA itself. This task can be accomplished with relative ease using the single cell electrophoresis (comet) assay (Figure 5). Undamaged genomic DNA from a single cell, when embedded in agarose and subjected to an electrical field, will migrate as an intact and roughly spherical mass. Conversely, when various forms of damage are sustained, the physical structure of the DNA will change (e.g., fragmented after ionizing radiation or crosslinked after exposure to platinum-based agents). The induction of such damage, as well as its repair can be observed and quantified using the comet assay.

A study by Wynne and colleagues adapted the comet assay to examine DNA repair in 50 ovarian cancer patients whose cells (derived at surgical resection or from ascites) were treated *ex vivo*

Control



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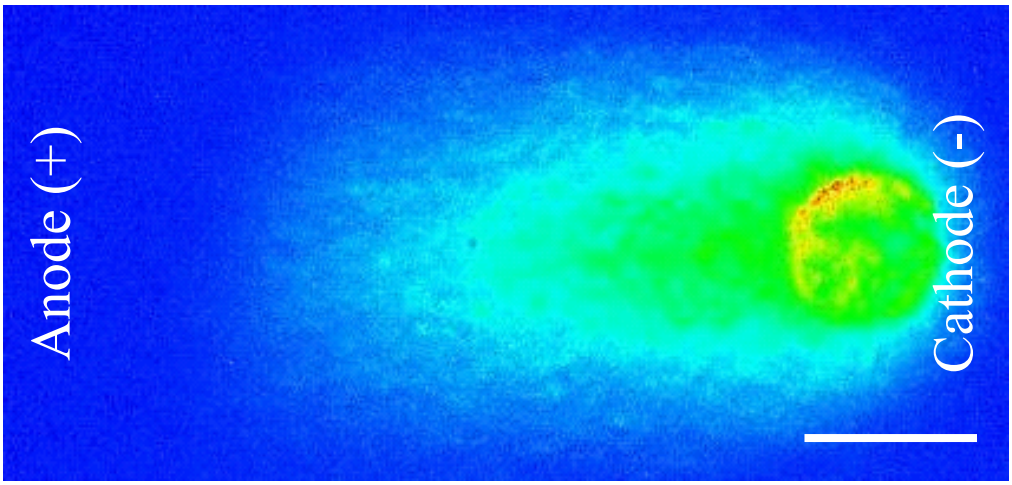


Figure 5. Single cell electrophoresis (comet) assay. MCF7 breast adenocarcinoma cells were exposed to zero or 10 Gy ionizing radiation from a ^{137}Cs source (5.46 $Gy \cdot min^{-1}$), trypsinized, and embedded in low melting point agarose. Cells were lysed *in situ* and electrophoresed at 1 V/cm for one hour, stained with ethidium bromide and visualized on an Olympus® fluorescence microscope. Pseudocoloring performed in Adobe® Photoshop.

with cisplatin. Thirty-six of the patients in this cohort were newly diagnosed and treatment-naïve, while 22 patients (some of whom were in the treatment-naïve cohort but had relapsed) had received previous chemotherapy. No differences in peak levels of crosslinking were found between the treatment-naïve and previously treated patients, however, at 24 hours post *ex vivo* exposure to cisplatin, crosslink repair was significantly higher in the group of previously treated patients (86% exhibited >10% repair and 64% demonstrated >50% repair compared to 36% and 3%, respectively, for the treatment-naïve group). For eight patients it was possible to acquire tumor samples prior to chemotherapy and at relapse. In these paired specimens, mean repair increased from 2.85% prior to chemotherapy to 71.23% in the specimens acquired after relapse, strongly arguing for DNA repair as a major mechanism in acquired chemotherapy resistance (Wynne et al., 2007). A notable advantage of the comet assay over assays designed to quantify molecular beacons of damage or repair is that the assay is not subject to hypostatic confounders. That is, the assay measures the fundamental consequence of repair and does not rely on interpretation of molecular signals in the context of genetic or epigenetic alterations that may or may not be significant. Indeed, the comet assay has been widely used to evaluate both risk and prognostication for diverse cancers as it is adept at detecting defects in pathways which ensure genomic stability without mechanistic assumptions as to the cause thereof (Berwick and Vineis, 2000).

Reversion Mutations and Acquired Resistance

Acquired therapeutic resistance remains a major clinical obstacle in oncology. In an effort to thwart the actions of chemotherapy and/or radiation, cancer cells are known to exploit membrane pumps to efflux drugs, inactivate apoptotic pathways, induce expression of detoxifying enzymes

and mutate the targets of antineoplastic agents. While it has been appreciated that increased DNA repair likely contributes to acquired chemoresistance to DNA damaging agents, two landmark studies published simultaneously demonstrated that *in vitro* selection of *BRCA2*-mutant ovarian and pancreatic cells resulted in outgrowth of cells that sustained intragenic frame restoring mutations at the *BRCA2* locus (Edwards et al., 2008; Sakai et al., 2008). To examine whether such a mechanism may confer resistance *in vivo*, Swisher and colleagues sequenced the *BRCA1* gene in nine recurrent *BRCA1*-mutated ovarian cancers previously treated with chemotherapy. Of these, one had primary resistance to platinum, five exhibited acquired platinum resistance, and three maintained sensitivity. Four of the six recurrent platinum-resistant tumors had sustained secondary intragenic changes in *BRCA1* that restored the *BRCA1* reading frame, whereas none of the platinum-sensitive tumors exhibited changes in *BRCA1* (Swisher et al., 2008). Another study by the same group examined *BRCA2* sequence changes in three syngeneic cell lines that were established prior to and after the onset of platinum resistance in a *BRCA2*-mutant ovarian cancer. The clone examined at the first relapse exhibited a *BRCA2* 5193C>G (Y1655X) mutation and was sensitive to cisplatin, whereas two clones isolated from ascitic fluid at the second relapse and in the terminal stages of the disease both exhibited a 5193C>T (Y1655Y) mutation that cancelled the effect of the inherited nonsense mutation, restored functional BRCA2 protein expression, and conferred resistance to platinum (Sakai et al., 2009). A very recent study by Swisher's group examined the frequency of secondary *BRCA1/2* mutations in recurrent *BRCA1/2*-associated ovarian cancers. This study evaluated 64 primary and 46 recurrent ovarian cancers and noted that 13 of the 46 (28%) recurrent tumors exhibited a secondary *BRCA1* or *BRCA2* mutation, and that these reversion events were nearly nine times more common in platinum-resistant recurrences than in those that remained platinum-sensitive.

Moreover, this study demonstrated that in women with recurrent ovarian cancer, those who had previously underwent chemotherapy for breast cancer also had higher frequency of secondary mutation in the recurrent ovarian lesion compared to those with no history of breast cancer (Norquist et al., 2011). These studies confirm that intragenic mutations in *BRCA1* and *BRCA2* are relatively frequent *in vivo* following chemotherapy of *BRCA1/2*-mutant ovarian cancers, argue that identification of such mutations can predict response to platinum-based agents, and strongly support a role for reversion mutation-induced repair-mediated resistance to platinum chemotherapy (Figure 6).

Genetic and Pharmacologic Targeting of the FA/BRCA Pathway

Many of the studies presented in this chapter support the idea that genetic or epigenetic interference of the FA/BRCA pathway sensitizes cells to DNA damaging agents that are commonly used in cancer therapy. This is reinforced by studies showing that hyperactivity, overexpression or mutational restoration of components of this pathway is associated with acquired resistance. Thus, genetic and/or pharmacologic inhibition of the FA/BRCA pathway may provide a new arsenal for the treatment of a number of malignancies, especially those that are refractory or exhibit acquired resistance to DNA damaging agents. An early study by Ferrer et. al. developed an adenoviral vector encoding a dominant-negative (DN) form of *FANCA* that interfered with the endogenous FANCA-FANCG interaction and ultimately impaired FANCD2 monoubiquitination. Infection of a number of cell lines with DN *FANCA* adenovirus sensitized cell lines 2-3 fold to cisplatin (Ferrer et al., 2004). Few studies have identified chemical agents that directly interfere with the FA/BRCA pathway. A screen conducted by Chirnomas et. al. identified that four compounds (wortmannin (PI3K inhibitor), H-9 (PKC, -G and –A inhibitor),

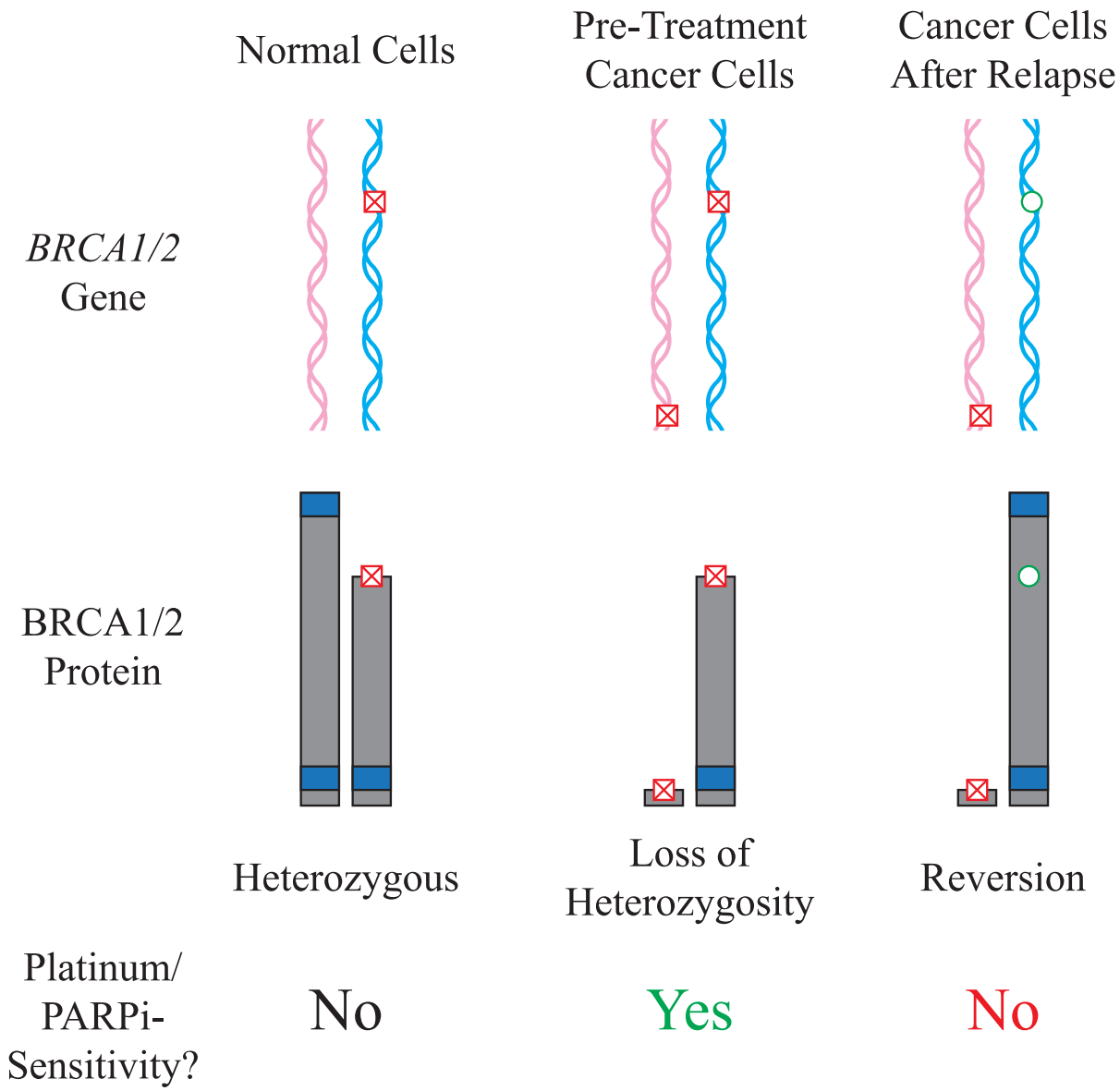

Normal Cells
Pre-Treatment Cancer Cells
Cancer Cells After Relapse
BRCA1/2 Gene
BRCA1/2 Protein
Heterozygous
Loss of Heterozygosity
Reversion
Platinum/ PARPi-Sensitivity?
No
Yes
No

Figure 6. Reversion mutations at the *BRCA1* and *BRCA2* loci can cause repair-mediated resistance.

alsterpaulone (GSK3β, CDK5/p25, and CDK1/Cyclin B inhibitor) and curcumin) were able to inhibit the FA/BRCA pathway. Specifically, curcumin, a natural compound found in turmeric, was able to inhibit the monoubiquitination of FANCD2 and sensitize breast and ovarian cancer cell lines to cisplatin by causing apoptosis (Chirnomas et al., 2006). Similarly, a study by Noguchi and colleagues reported impaired homologous recombinational repair of cancer cells treated with the heat shock protein 90 (HSP90) inhibitor 17-AAG that is currently in active clinical trials. This study demonstrated that 17-AAG destabilized BRCA2 and in effect altered RAD51 expression and function (Noguchi et al., 2006). Lastly, a study by Landais et. al. screened a chemical library for inhibitors of *Xenopus* FANCD2 monoubiquitination and identified 2,3-dichloro-5,8-dihydroxy-1,4-naphthoquinone (DDN) as a novel potent inhibitor of the FA pathway. DDN inhibited FANCD2 monoubiquitination of both human and Xenopus systems, inhibited subnuclear assembly of FANCD2 into foci, and displayed a synergistic effect with cisplatin in FA-proficient cancer cells (Landais et al., 2009). Given the potential of synthetic lethal approaches, it is interesting to speculate that PARP inhibitors combined with novel pharmacologic inhibitors of the FA/BRCA pathway may prove to be highly effective anti-neoplastic regimens.

Discussion

Our mechanistic understanding of DNA repair has benefited remarkably from studying Fanconi anemia and hereditary breast and/or ovarian cancer. While inherited and acquired defects in the genes of the FA/BRCA pathway compromise genomic integrity and incite malignancy, these same alterations may represent an Achilles' heel in tumors cells. Perhaps the same genetic instability that epitomizes cancer and allows its incessant phenotypic evolution is also among its

most exploitable vulnerabilities. The immediate challenge is to identify, at the level of each patient, those tumors which exhibit compromised FA/BRCA pathway function and to tailor cytotoxic chemotherapy accordingly. The relative frequency of acquired genetic and/or epigenetic alterations in this pathway suggests that double-strand break and interstrand crosslink repair are legitimate targets for therapeutic intervention or exploitation. Moreover, novel therapeutic strategies aimed at crippling the activity of this pathway may show promise in preventing repair-mediated resistance to current cytotoxic agents. A comprehensive understanding of FA/BRCA dysfunction in human cancers of both hereditary and sporadic nature, coupled with advances in diagnostic, genetic and pharmacologic tools to evaluate and manipulate expression or function of this pathway are certain to improve the clinical outcomes for patients with a number of cancers.

In the coming chapters, we provide compelling evidence that BRCA1 is a novel client protein of HSP90 and that the clinically viable HSP90 inhibitor 17-AAG can regulate BRCA1-dependent repair of DSBs and ICLs. These studies illuminate a novel mechanism to combat repair-mediated resistance to platinum-based chemotherapy and ionizing radiation in breast, ovarian and potentially other malignancies. We also report preliminary data on utilizing *BRCA1* promoter methylation as a predictive biomarker for response to anthracycline therapy in triple-negative breast cancer, and speculate that platinum-based agents may yield more favorable clinical responses in a subset of BRCA1-deficient sporadic TNBCs.

Chapter II: BRCA1 Stability and Function is Regulated by HSP90

Introudction

Inherited mutations in the breast cancer susceptibility gene 1 (*BRCA1*) predispose to the development of breast, ovarian, and other malignancies (Ford et al., 1994; Friedman et al., 1994; Hall et al., 1990; King et al., 2003; Thompson et al., 1995). BRCA1 is a nuclear phosphoprotein with broad tumor suppressor activities and is known to play a critical role in the repair of double-strand DNA breaks (DSBs) and interstrand crosslinks (ICLs) by homologous recombination (HR) (Huen et al.). Upon induction of DSBs or ICLs, BRCA1 is phosphorylated by ATM, ATR, and CHK2 kinases and appears to recruit and organize multiple distinct protein complexes that recognize and repair damaged DNA and activate cell cycle checkpoints (Cortez et al., 1999; Yarden et al., 2002).

Therapeutic strategies based on synthetic lethality have emerged that target cells deficient in DSB repair (reviewed in Chapter I). These approaches exploit the fact that inherited or acquired defects in a single pathway, such as BRCA1-dependent DSB repair, may have few or no deleterious effects on cellular function due to the action of another redundant or adaptive pathway. However, simultaneous inactivation of an ancillary pathway that rescues DSB repair-deficient cells will lead to accumulation of unrepaired DNA damage and cell death. Specifically, the DSB repair-deficient cancers arising in *BRCA1* mutation carriers are highly sensitive to inhibitors of poly(ADP-ribose) polymerase (PARP), an enzyme critical in base excision repair (Adhikari et al., 2008). Clinical trials employing PARP inhibitors are currently ongoing and these agents show promise in the treatment of *BRCA1* and *BRCA2*-associated breast, ovarian and prostate cancers, as well as sporadic basal-like breast cancers, which are thought to have

dysfunction of the BRCA1 pathway in the absence of mutations at its genetic locus (Fong et al.; Turner et al., 2007).

In light of the body of evidence suggesting that high BRCA1 expression in both breast and ovarian cancers contributes to therapeutic resistance, there has been much interest in identifying agents which interfere with BRCA1-dependent DNA damage repair. In the current study, we have identified BRCA1 as a novel client protein of heat shock protein 90 (HSP90) and demonstrate that pharmacologic inhibition of HSP90 using 17-allylamino-17-demethoxygeldanamycin (17-AAG) results in profound loss of BRCA1 expression and function. HSP90 is an evolutionarily conserved chaperone that promotes the proper folding of client proteins, thereby regulating their stability, expression, trafficking and function (Whitesell and Lindquist, 2005). Simultaneous disruption of signaling nodes that regulate all hallmarks of cancer can be achieved by inhibition of HSP90 (Blagg and Kerr, 2006; Hanahan and Weinberg, 2000). Natural compounds including geldanamycin, radicicol and novobiocin have been identified to bind the N- or C-terminal ATP-binding pockets of HSP90 and disrupt its chaperone function, leading to client protein degradation via the ubiquitin-proteasome pathway (Blagg and Kerr, 2006). Semi-synthetic analogues of geldanamycin, including 17-AAG, as well as newer generation HSP90 inhibitors are undergoing clinical trials for the treatment of a variety of human malignancies (Kim et al., 2009). These inhibitors have shown promise in sensitizing tumor cells to numerous genotoxic agents which are commonly used in cancer therapy, including DNA alkylating agents, ionizing radiation, DNA replication inhibitors and PARP inhibitors (Camphausen and Tofilon, 2007; Dungey et al., 2009).

The ability of HSP90 inhibitors to potentiate cells to ionizing radiation and chemotherapeutic agents can be explained by the critical role of HSP90 in stabilizing and/or activating components of the HR/ICLR and non-homologous end joining (NHEJ) DSB repair machinery, including CHK1, BRCA2, RAD51, FANCA, and DNA-PKcs (Arlander et al., 2003; Dote et al., 2006; Noguchi et al., 2006; Oda et al., 2007). Except for DNA-PKcs, the activity and/or recruitment of these molecules to sites of DNA damage is dependent on BRCA1 function (Bhattacharyya et al., 2000; Chen et al., 2008; D'Andrea and Grompe, 2003; Folias et al., 2002; Yarden et al., 2002; Zhang et al., 2009; Zhong et al., 1999). We report here that pharmacologic inhibition of HSP90 results in rapid loss of BRCA1 through the ubiquitin-proteasome pathway, subsequent failure of BRCA1 to assemble at ionizing radiation-induced foci (IRIF), and functional defects in both HR and NHEJ. In summary, while previous studies have documented defects in HR and NHEJ as a consequence of inhibiting HSP90, we provide direct evidence that loss of BRCA1 following inhibition of HSP90 is a key event leading to defective DSB repair and potentiation to DNA damaging agents.

Materials and Methods

Cell Culture

All cell lines were obtained from and cultured according to ATCC recommendations.

Adenoviruses and Adenoviral Production

The coding region of the I-SceI endonuclease with two 5' nuclear localization signals and a hemagglutnin (HA) tag was excised from the pCBASce plasmid as an XbaI/MscI fragment and cloned into pShuttle-CMV which had been digested with XbaI and EcoRV. The pShuttle-CMV-

I-SceI plasmid was then digested with PmeI, desalted by Qiaex, and then electroporated into *E. coli* strain BJ5183 along with pAdEasy1 to allow homologous recombination to occur (He et al., 1998). The resultant vector was PacI linearized and was transfected into AD293 cells. Adenoviral supernatants were produced by freeze/thaw and sonication and were titered using the Adeno-X Rapid Titer Kit (Clontech). The HA-tagged wild-type and deletion mutant adenoviruses (Chiba and Parvin, 2002) were a kind gift from Jeffrey Parvin (The Ohio State University Medical Center) and were produced as describe above.

Lentivirus Generation and Infection

The Expression Arrest™ GIPZ lentiviral shRNAmiR library targeting BRCA1 and control GIPZ vector were purchased from OpenBiosystems (ThermoFisher Scientific, Waltham, MA). Lentiviruses were packaged in HEK293T cells using pRC-CMV-RaII, pHDM-tat16, pHDM-HG-PM2, and pHDM-VSVG packaging constructs. Supernatants were ultracentrifuged and infectious particles were titered by flow cytometry according to the equation $= \frac{P \cdot N}{D \cdot V}$, where T equals titer in transducing units (TU) per mL, P equals the percentage of GFP^+ cells, N equals the number of cells at the time of transduction, D equals the dilution of virus, and V equals the volume of viral inoculum in mL. MCF7 cells were infected at a multiplicity of infection (MOI) of 0.2 according to the equation $V = \frac{C \cdot MOI}{T}$, where V equals the volume of stock virus required in mL, C equals the number of cells being infected, MOI equals the desired multiplicity of infection, and T equals titer (in TU/mL) calculated as described above. Cells were selected for two weeks in 2 µg/mL puromycin and were maintained in selective media for the duration of all experiments.

Drug Treatment

17-AAG was purchased from InvivoGen, radicicol was purchased from Tocris, novobiocin, cycloheximide, carboplatin and MG132 were purchased from Sigma-Aldrich. Carboplatin was dissolved in PBS, all other drugs were dissolved in DMSO.

Irradiation

Cells were irradiated using a ^{137}Cs source irradiator at a dose rate of 4.97 $Gy \cdot min^{-1}$.

DNA Foci Formation Assay

Cells were grown in 8-well chamber slides, treated and exposed to 0 or 10 Gy ionizing radiation. Fixative was 4% paraformaldehyde with 3% sucrose on ice for 30 minutes. Permeabilizer was 0.1% Triton X-100 in PBS for five minutes at room temperature. Cells were blocked with blocking buffer (1% bovine serum albumin (fraction V), 3% goat serum, and 0.1% Triton X-100) for two hours at 4°C. Primary antibodies were diluted in blocking buffer (BRCA1 Ab1 (1:200), γH2AX (1:1000)) and incubated on fixed/permeabilized cells overnight at 4°C. Secondary antibody was goat anti-mouse Alexa 594 (Invitrogen/Molecular Probes) diluted in blocking buffer at 1:1000. Nuclei were counterstained with DAPI (Sigma-Aldrich) at 50 ng/mL for thirty minutes. Foci formation was quantitated using the Zeiss Pascal confocal software by normalizing the fluorescent signal for γH2AX or BRCA1 to the DAPI signal.

Homologous Recombination and Non-Homologous End Joining

The DR-GFP reporter construct, pCBASce (I-SceI) and pCAGGS (empty control) vectors were kindly provided by Maria Jasin (Memorial Sloan-Kettering Cancer Center). This assay was

performed essentially as previously described (Nakanishi et al., 2005). Briefly, HeLa cells were transfected with the DR-GFP reporter plasmid, selected with 5 µg/mL puromycin, and cloned. The I-SceI or empty control vector were electroporated into the HeLa-DR-GFP cells (1 µg/1x10^5) cells using the Amaxa nucleofector system (Lonza, Walkersville, MD) and cells were immediately plated into 250 nM 17-AAG or DMSO. After 24 hours, the drug was removed and replaced with fresh media for an additional 24 hours. Cells were trypsinized and HR was measured by assessing number of GFP+ cells on a BD LSR II flow cytometer. Genomic DNA was isolated from a fraction of the treated cells using the DNeasy Blood and Tissue Kit from Qiagen and used as the template for PCR with the following primers: DRGFP-F, 5'-CTGCTAACCATGTTCATGCC-3' and DRGFP-R, 5'-AAGTCGTGCTGCTTATGTG-3'. PCR conditions were one cycle for one minute at 94°C for initial denaturation; 35 cycles for 20 seconds at 94°C for denaturation, then 40 seconds at 54°C for annealing, and 40 seconds at 72°C for extension; and one cycle for 7 minutes at 72°C for final extension. PCR products were purified using the QIAquick Purification Kit (Qiagen) and then digested with 10 units of I-SceI (New England Biolabs) for 16 hours at 37°C and then heat inactivated at 65°C for 20 minutes. A half-volume of the product was subsequently digested with 20 units of BcgI for four hours at 37°C and then heat inactivated at 65°C for 20 minutes. Samples were electrophoresed on a 1.2% agarose gel in 1X TAE and the gel was stained with ethidium bromide. Digested products were visualized and quantitated using the UVP BioImaging System (UVP).

Antibodies

Mouse anti-Actin (#MAB1501, Upstate/Millipore), mouse anti-BARD1 (#ab50984, Abcam), mouse anti-BRCA1 (Ab1) (#OP92, Calbiochem/EMD Biosciences), rabbit anti-BRCA1 (C-20)

(#sc-642, Santa Cruz Biotechnology), mouse anti-phospho-Histone H2A.X (Ser139) (γH2AX) (#05-636, Upstate/Millipore), mouse anti-HSP90 (#05-594, Upstate/Millipore), mouse anti-HSP90 (#ADI-SPA-830, Stressgen), mouse anti-Ubiquitin (#13-1600, Invitrogen), anti-HA (#H3663, Sigma-Aldrich), anti-rabbit HRP-linked (#7074, Cell Signaling Technology) and anti-mouse HRP-linked (#7076, Cell Signaling Technology).

Immunoprecipitation and Western Blot

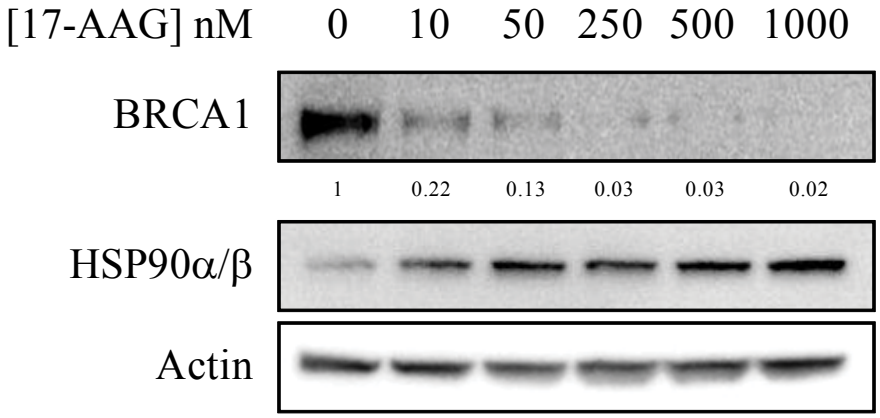
Protein was harvested using a modified RIPA buffer (50 mM Tris base, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM NaF, 5 mM Na_3VO_4, plus 1X protease inhibitor cocktail (Sigma-Aldrich)), quantified using the D_C protein assay (Bio-Rad), electrophoresed on 8% or 4-12% tris-glycine gels (Invitrogen) and then transferred onto PVDF membranes. All blocking steps and antibody-dilutions were in 3% non-fat dairy milk in PBS + 0.1% Tween-20. Densitometry calculations were made using the UVP BioImaging System. For IP, cells were lysed in IP buffer (20 mM Tris-HCl (pH 7.5-7.9), 100 mM NaCl, 0.05% NP-40, 1 mM PMSF), immunoprecipitated with 10 μg antibody overnight at 4°C on a rotating platform. Complexes were bound to Protein-A agarose beads (#15918-014, Invitrogen), washed and eluted according to manufacturer's protocol.

Results

HSP90 Interacts with BRCA1 and is Necessary for its Stability

To understand the regulatory properties of HSP90 on BRCA1 expression, we examined the effects of 17-AAG treatment on BRCA1 in MCF7 breast cancer cells. 17-AAG downregulated BRCA1 in a dose- and time-dependent manner (Figure 1A-B), and when combined with

A


[17-AAG] nM 0 10 50 250 500 1000
BRCA1
1 0.22 0.13 0.03 0.03 0.02
HSP90α/β
Actin

B

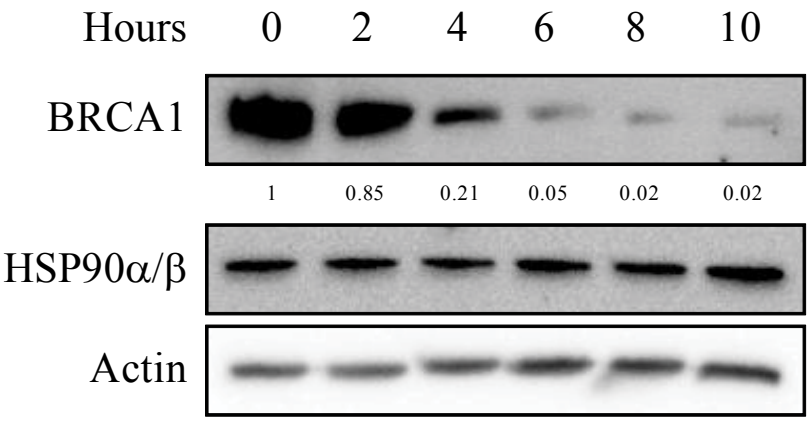

Hours 0 2 4 6 8 10
BRCA1
1 0.85 0.21 0.05 0.02 0.02
HSP90α/β
Actin

Figure 1. Inhibition of HSP90 induces degradation of BRCA1. (A) Western blots of MCF7 cells treated with indicated concentrations of 17-AAG for eight hours. (B) Western blots of MCF7 cells treated with 250 nM 17-AAG for indicated duration.

cycloheximide, increased the rate of BRCA1 decay in HCC1937^{BRCA1} cells beyond that observed with cycloheximide alone (Figure 2). Transcriptional activation of the *BRCA1* promoter and levels of BRCA1 mRNA showed delayed effects, with a marginal increase in expression of *BRCA1* promoter-driven luciferase 24 hours post treatment and a progressive loss of reporter transactivation by 48 and 72 hours (Figure 3). Furthermore, BRCA1 protein degradation preceded loss of BRCA1 mRNA (Figure 4), suggesting that destabilization of BRCA1 protein is likely the major mechanism for BRCA1 loss in response to 17-AAG, though transcriptional downregulation may contribute to loss of BRCA1 at later timepoints. 17-AAG treatment also increased expression of HSP90 mRNA and protein (Figure 1A-B and 4), reflecting a previously described feedback stimulation of stress-responsive genes (McCollum et al., 2008). 17-AAG treatment did not simultaneously alter expression of the constitutively associated BRCA1-Associated Ring Domain 1 (BARD1) protein, which is necessary for BRCA1 stability and function (Figure 5) (Joukov et al., 2001). Kinetic studies in SK-BR-3 ($HER2^+$ breast cancer cell line), MDA-MB-231 (triple-negative breast cancer cell line), SK-OV-3 (ovarian cystadenocarcinoma cell line) and HT-29 (colorectal adenocarcinoma cell line) cells revealed that 17-AAG induced degradation of BRCA1 expression in all cell lines tested (Figure 6A). Treatment of MCF7 cells with radicicol and novobiocin, two additional inhibitors of HSP90 chaperone function, also resulted in loss of BRCA1 (Figure 6B).

These data suggest that BRCA1 may be a client protein of HSP90. Co-immunoprecipitation studies revealed that BRCA1 and HSP90 interact at basal levels, that irradiation of cells increases the association between BRCA1 and HSP90, and that treatment with 17-AAG can abolish this interaction (Figure 7). We were unable to reproducibly demonstrate the interaction

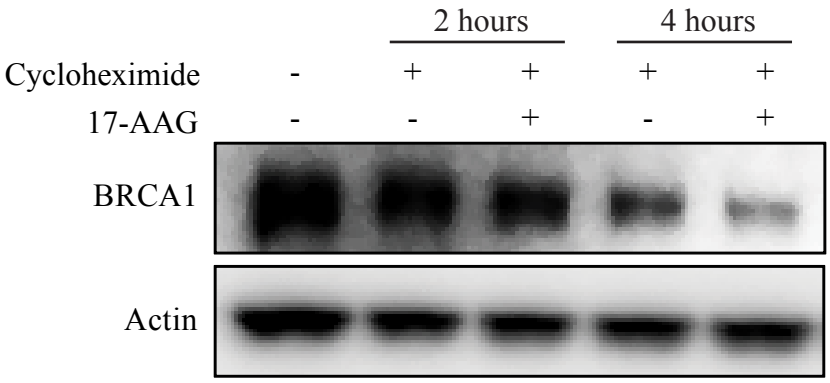

2 hours
4 hours
Cycloheximide
-
+
+
+
+
17-AAG
-
-
+
-
+
BRCA1
Actin

Figure 2. 17-AAG decreases the endogenous half-life of BRCA1. Western blot of HCC1937^{BRCA1} cells treated with 25 μg/mL cycloheximide in the presence or absence of 1 μM 17-AAG for the indicated duration.

BRCA1 Promoter-Luciferase

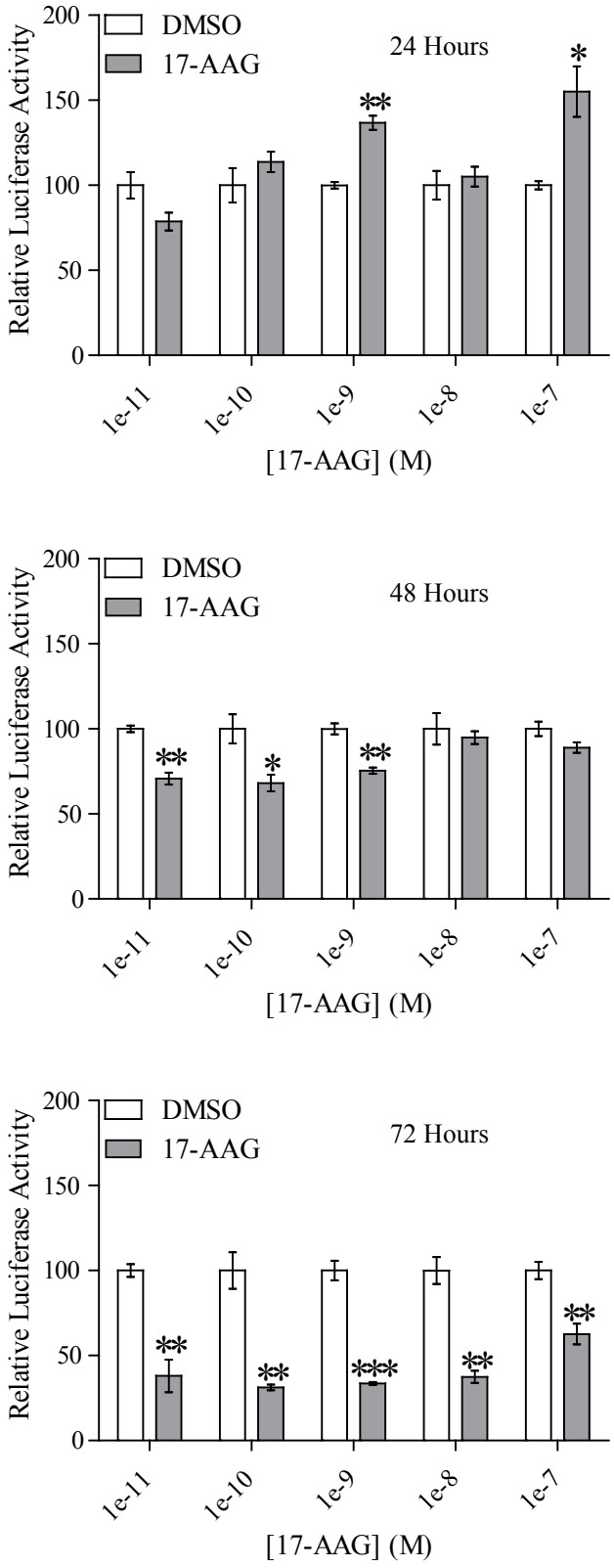


Figure 3. Inhibition of Hsp90 leads to a delayed repression of the BRCA1 promoter. MCF7 cells with a stably-integrated BRCA1 promoter-driven Firefly luciferase reporter were treated with the indicated dose of 17-AAG for 24, 48, or 72 hours. Each timepoint was normalized such that the average DMSO luciferase activity is 100%. Error bars represent SEM of triplicate measurements. *$p<0.05$, **$p<0.01$, ***$p<0.001$, student's t-test.

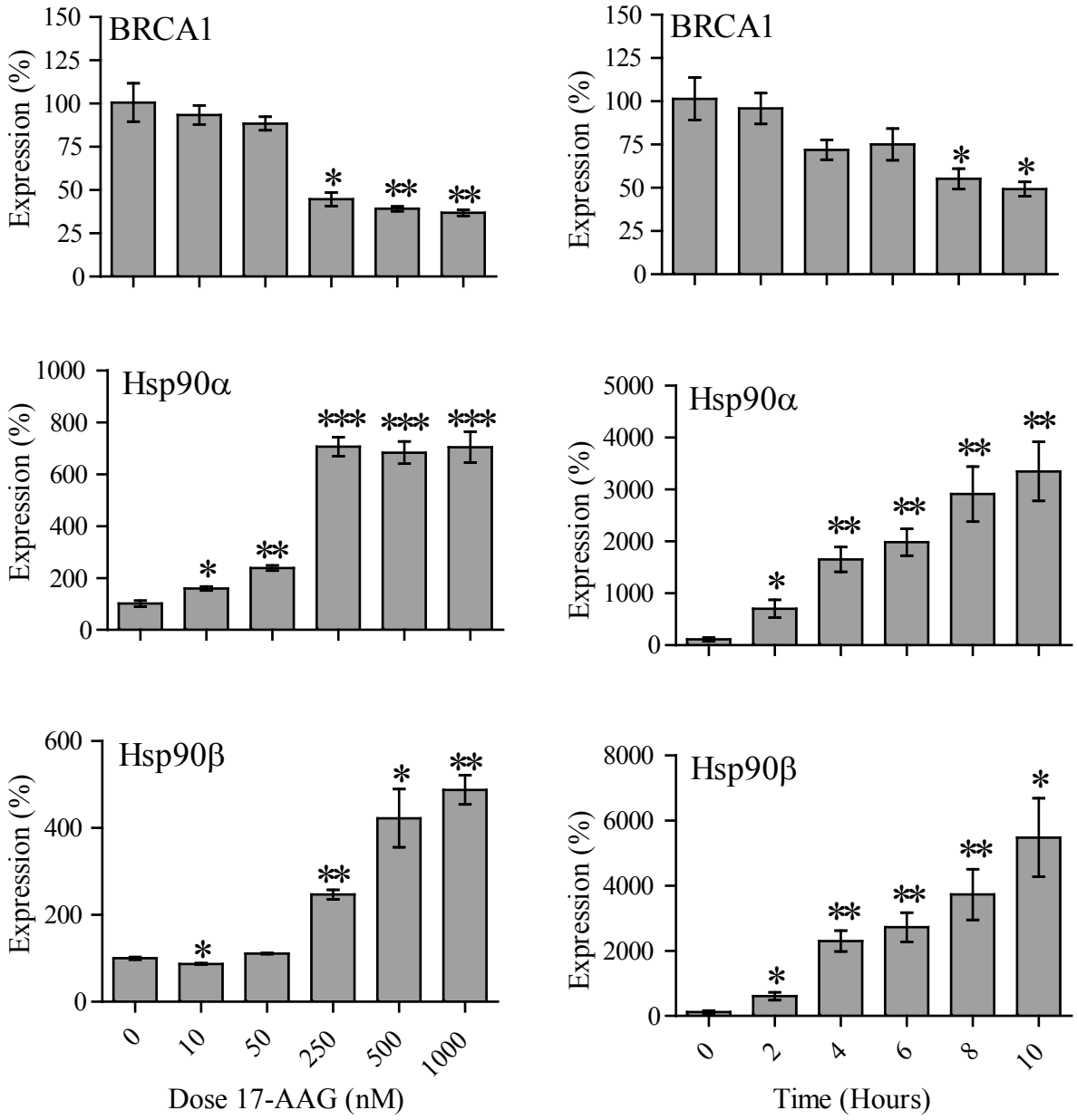

BRCA1
Expression (%)
150
125
100
75
50
25
0
*
**
**
BRCA1
Expression (%)
150
125
100
75
50
25
0
*
*
Hsp90α
Expression (%)
1000
800
600
400
200
0
*
**
***
***
***
Hsp90α
Expression (%)
5000
4000
3000
2000
1000
0
*
**
**
**
**
Hsp90β
Expression (%)
600
400
200
0
*
**
*
**
0
10
50
250
500
1000
Dose 17-AAG (nM)
Hsp90β
Expression (%)
8000
6000
4000
2000
0
*
**
**
**
*
0
2
4
6
8
10
Time (Hours)

Figure 4. Inhibition of Hsp90 leads to a delayed loss of BRCA1 mRNA. MCF7 cells were treated with the indicated dose of 17-AAG for eight hours (left column) or with 250 nM 17-AAG for the indicated duration (right column) and total cellular RNA was isolated and utilized for qRT-PCR analysis of BRCA1, HSP90α and HSP90β mRNA expression. Error bars represent SEM of triplicate measurements. *$p<0.05$, **$p<0.01$, ***$p<0.001$, student's t-test.

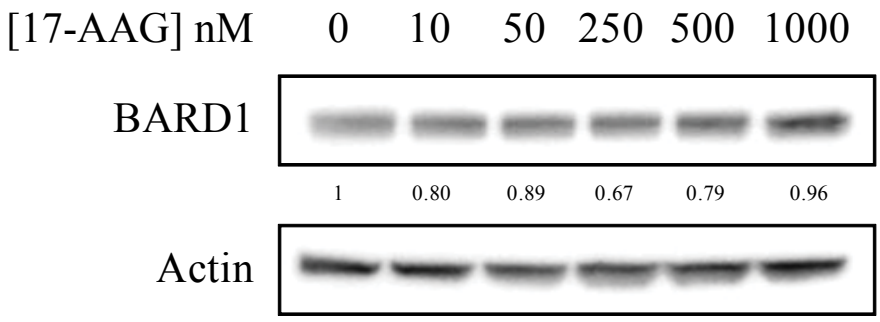

[17-AAG] nM
0
10
50
250
500
1000
BARD1
1
0.80
0.89
0.67
0.79
0.96
Actin

Figure 5. 17-AAG does not simulateneously destabilize BARD1. Western blots of MCF7 cells treated with indicated concentrations of 17-AAG for eight hours.

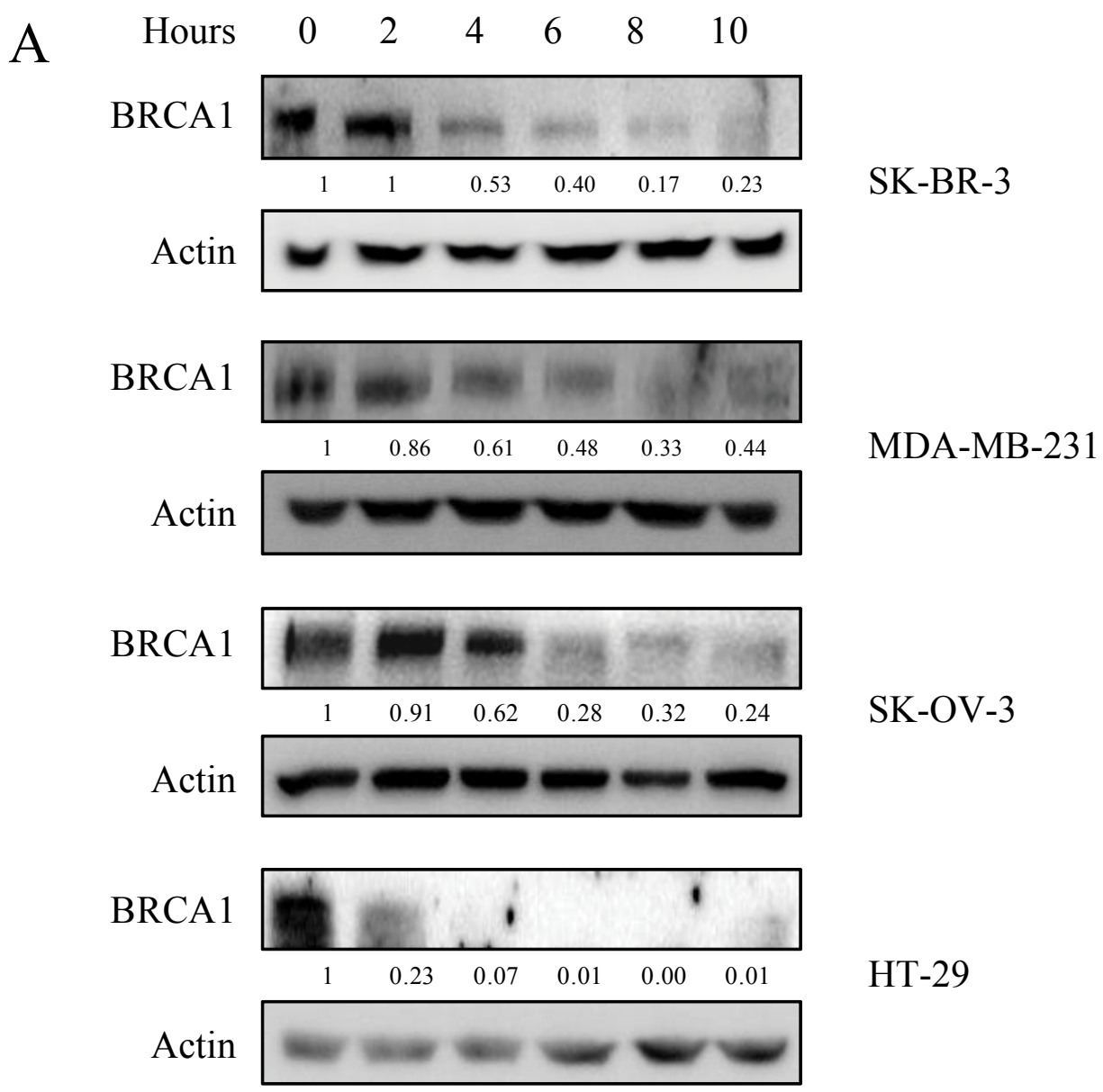

A
Hours 0 2 4 6 8 10
BRCA1
1 1 0.53 0.40 0.17 0.23
SK-BR-3
Actin
BRCA1
1 0.86 0.61 0.48 0.33 0.44
MDA-MB-231
Actin
BRCA1
1 0.91 0.62 0.28 0.32 0.24
SK-OV-3
Actin
BRCA1
1 0.23 0.07 0.01 0.00 0.01
HT-29
Actin
B
DMSO
17-AAG
Radicicol
Novobiocin
BRCA1
Actin

Figure 6. 17-AAG indcues BRCA1 degradation in multiple cell lines and multiple HSP90 inhibitors are able to destabilize BRCA1. (A) Western blots of SK-BR-3 and MDA-MB-231 breast cancer cells, SK-OV-3 ovarian cancer cells, and HT-29 colorectal cancer cells treated with 250 nM 17-AAG for the indicated duration. (B) Western blots of MCF7 cells treated with DMSO, 250 nM 17-AAG, 2 μM radicicol, or 2 mM novobiocin for eight hours.

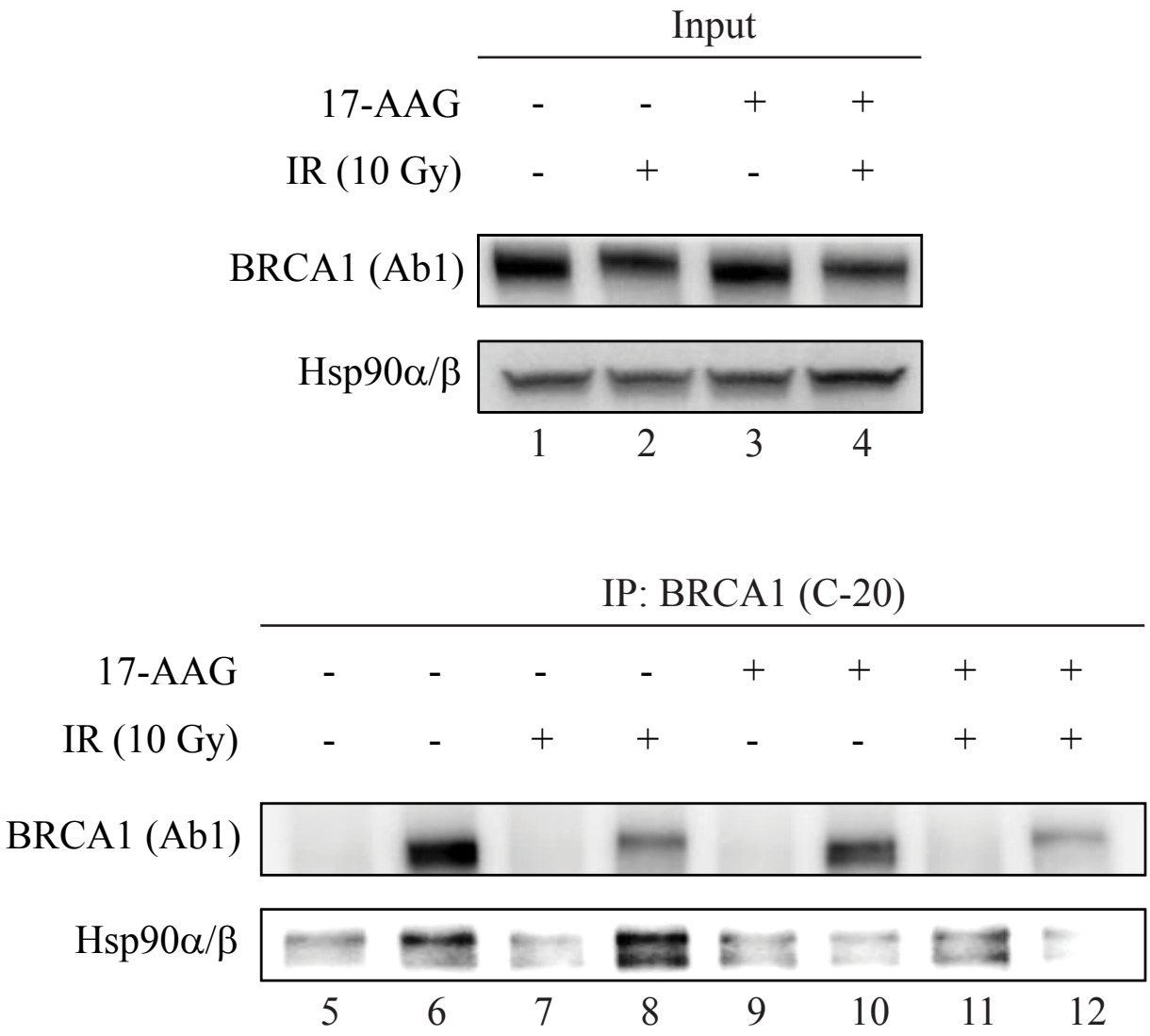

Input
17-AAG
- - + +
IR (10 Gy)
- + - +
BRCA1 (Ab1)
Hsp90α/β
1 2 3 4
IP: BRCA1 (C-20)
17-AAG
- - - - + + + +
IR (10 Gy)
- - + + - - + +
BRCA1 (Ab1)
Hsp90α/β
5 6 7 8 9 10 11 12

Figure 7. The interaction between BRCA1 and HSP90 can be enhanced by genotoxic stress and abolished by 17-AAG treatment. MCF7 cells were treated with 250 nM 17-AAG with or without exposure to 10 Gy IR for four hours and then were immunoprecipitated with rabbit anti-BRCA1 (C-20) (lanes 6, 8, 10 and 12) or with control rabbit IgG (lanes 5, 7, 9 and 11). Lanes 1-4 are 1/25 input. Proteins were electrophoresed and probed with mouse anti-BRCA1 (Ab1) or mouse anti-HSP90.

in the opposite direction, implying that the bulk of cellular BRCA1 may not be constitutively associated with HSP90. To evaluate functional regions that may be important in mediating 17-AAG-induced BRCA1 degradation, we evaluated the effect of 17-AAG on MCF7 cells infected with HA-tagged BRCA1 wild-type and deletion mutant expressing adenoviruses. We observed that amino acids 775-1292 are likely important in mediating 17-AAG-induced BRCA1 degradation, as exogenously expressed protein lacking this domain appeared to be stable in the presence of 17-AAG (Figure 8). Assessment of the effect of 17-AAG on a large N-terminal truncation (Δ1-302) was not possible, as this mutant is unable to bind to BARD1 and would be expected to be highly unstable (Figure 8) (McCarthy et al., 2003). Inhibition of HSP90 induces polyubiquitination and proteasomal degradation of proteins that are dependent on HSP90 chaperone function (Blagg and Kerr, 2006). To examine ubiquitination of BRCA1 in response to 17-AAG, we treated MCF7 cells with 10 μM of the 26S proteasome inhibitor MG132, 250 nM 17-AAG, or both for two hours and immunoprecipitated BRCA1. This duration of 17-AAG treatment alone had no detectable effect on BRCA1 levels (consistent with data in Figure 1), while treatment with MG132 alone resulted in mild accumulation of polyubiquitinated BRCA1, supporting previous observations that the ubiquitin-proteasome pathway is involved in regulating basal levels of BRCA1 (Figure 9A) (Choudhury et al., 2004). Simultaneous treatment with 17-AAG and MG132 led to robust accumulation of polyubiquinated BRCA1 (Figure 9A). We confirmed that pre-treatment with MG132 for one hour followed by 250 nM 17-AAG for an additional eight hours partially rescued BRCA1 from degradation (Figure 9B). Taken together, these results suggest that the chaperone activity of HSP90 is required to maintain expression of BRCA1 and that inhibition of HSP90 activity induces the polyubiquitination and subsequent proteasomal degradation of BRCA1.

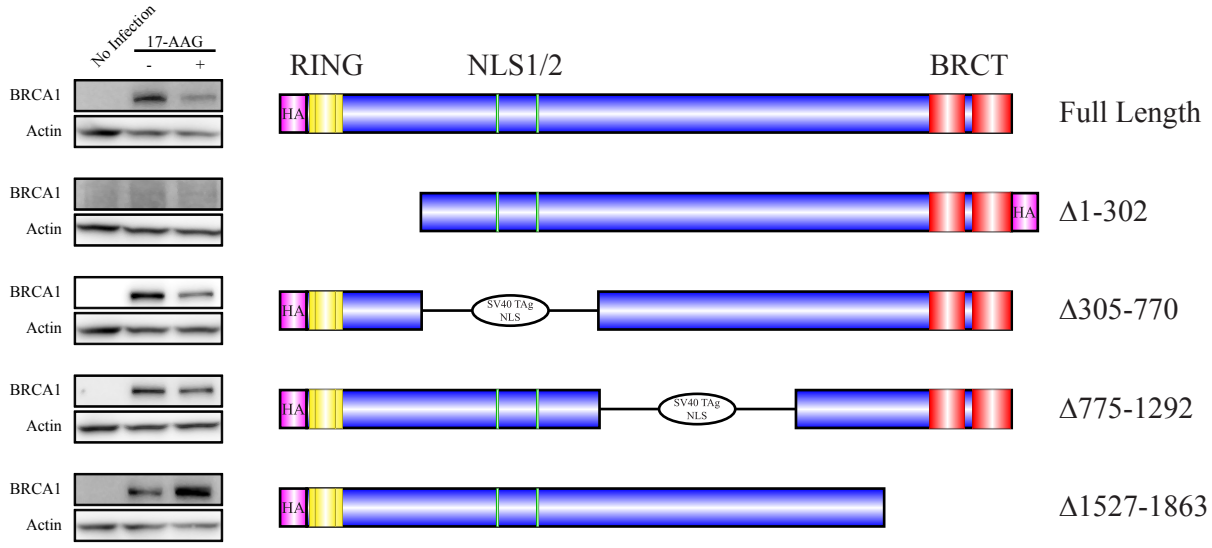
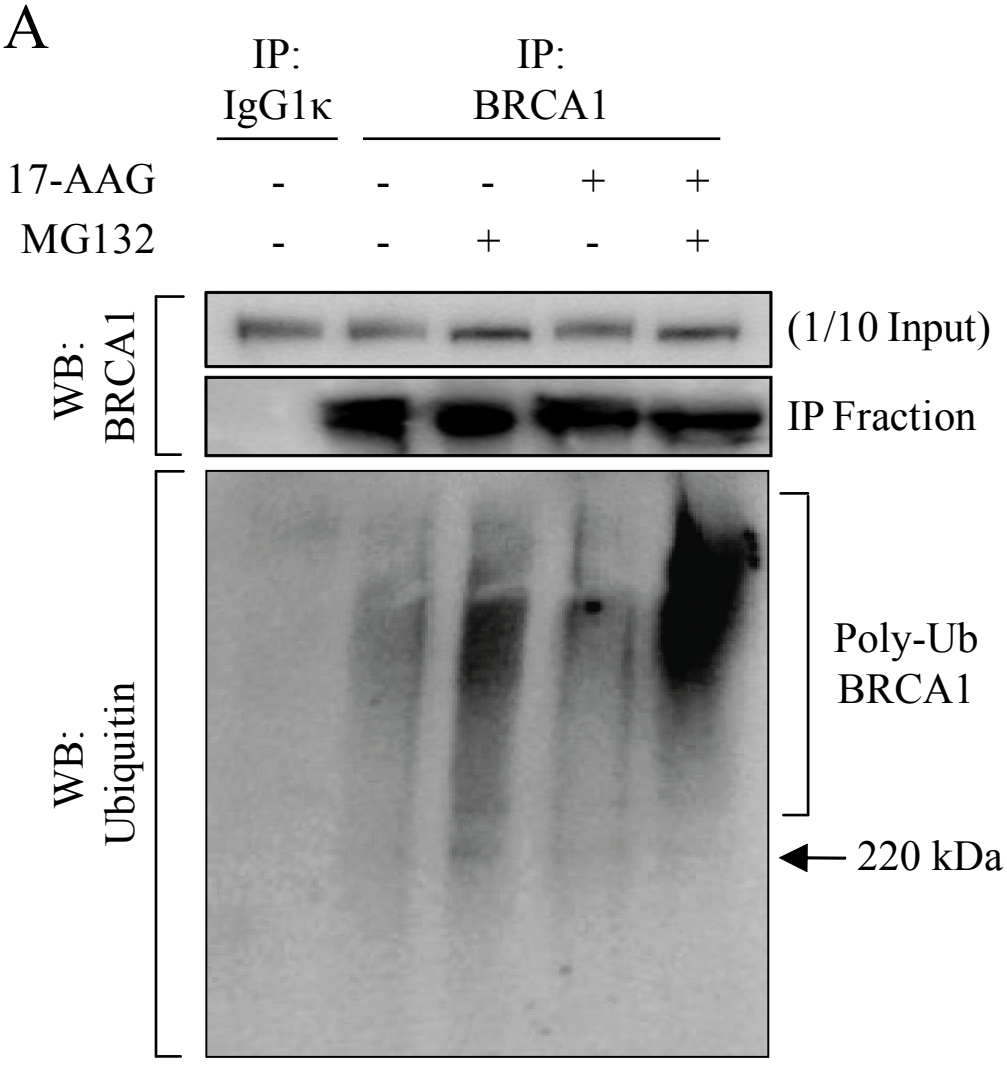

No Infection
17-AAG
-
+
BRCA1
Actin
BRCA1
Actin
BRCA1
Actin
BRCA1
Actin
BRCA1
Actin
RING
NLS1/2
BRCT
HA
Full Length
HA
Δ1-302
HA
SV40 TAg NLS
Δ305-770
HA
SV40 TAg NLS
Δ775-1292
HA
Δ1527-1863

Figure 8. Identification of functional domains in BRCA1 important for 17-AAG-induced BRCA1 degradation. MCF7 cells were infected with BRCA1 wild-type or indicated deletion mutant-encoding adenoviruses at an MOI of 250. After 48 hours, 1 μM 17-AAG or vehicle was added for eight hours and total protein was used for Western blot.

A


IP:
IgG1κ
IP:
BRCA1
17-AAG - - - + +
MG132 - - + - +
WB:
BRCA1
(1/10 Input)
IP Fraction
WB:
Ubiquitin
Poly-Ub
BRCA1
220 kDa

B

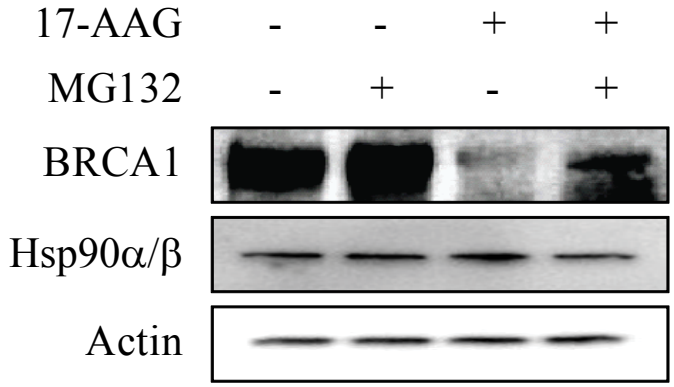
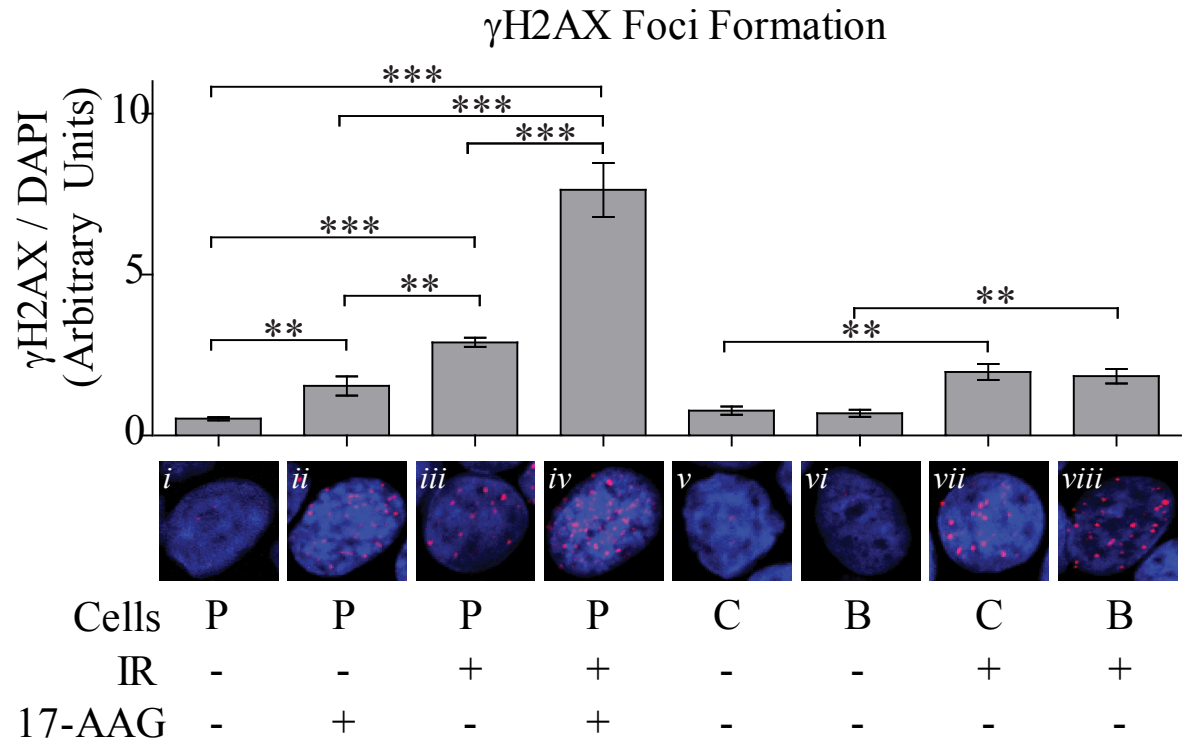

17-AAG - - + +
MG132 - + - +
BRCA1
Hsp90α/β
Actin

Figure 9. 17-AAG induces polyubiquitination and proteasome-mediated degradation of BRCA1. (A) MCF7 cells were treated with vehicle (-) or 250 nM 17-AAG in the presence or absence of 10 μM MG132 for two hours. Cells were lysed and a BRCA1 monoclonal antibody or isotype control antibody was used to immunoprecipitate BRCA1. Proteins were electrophoresed and probed for BRCA1 (top two blots) or with a ubiquitin antibody (bottom blot) to detect polyubiqiuitinated BRCA1. (B) Western blots of MCF7 cells pretreated with vehicle (-) or 10 μM MG132 for one hour and then treated with additional vehicle or 250 nM 17-AAG for eight hours.

Inhibition of HSP90 Impairs Assembly of BRCA1 to Ionizing Radiation-Induced Foci

Recruitment of BRCA1 to sites of DNA damage is a dynamic process involving a large number of proteins (Shrivastav et al., 2008). Induction of DSBs leads to the rapid phosphorylation of histone H2AX by ATM (γH2AX) (Burma et al., 2001). This phosphorylation event induces ubiquitination of γH2AX and subsequent recruitment of a number proteins required for the stability and assembly of the DSB repair complex, including RAP80, ABRA1, MERIT40, BRCC36, BRCC45, BRCA1, and BARD1 (Huen et al.). To assess whether inhibiting HSP90 affects upstream DSB signaling and/or assembly of BRCA1 to sites of DNA damage, we treated MCF7 cells with DMSO or 250 nM 17-AAG for 24 hours, exposed them to 10 Gy of IR and immunostained for γH2AX and BRCA1. MCF7 cells infected with lentiviruses expressing control or BRCA1-targeting shRNA were included as controls. Treatment with 17-AAG prior to IR did not prevent, but rather augmented γH2AX foci formation (Figure 10A, compare *iii* and *iv*) but significantly interfered with assembly of BRCA1 to IRIF (Figure 10B, compare *iii* and *iv*). Interestingly, treatment with 17-AAG in the absence of IR also induced γH2AX foci formation (Figure 10A, compare *i* and *ii*), suggesting that 17-AAG may lead to accumulation of spontaneous DSBs by inhibiting basal DSB repair. This effect of 17-AAG is likely independent of its effect on BRCA1 expression, as shRNA-mediated BRCA1 ablation did not increase γH2AX foci formation above baseline (Figure 10A, compare *v* and *vi*). These results demonstrate that HSP90 inhibition causes failed localization of BRCA1 to sites of DSBs while simultaneously causing accumulation of DSBs and/or augmenting early signaling events associated with DSB induction. Importantly, while the expression or localization of other components of the HR DSB repair complex are known to be affected by inhibiting HSP90

A


γH2AX Foci Formation
γH2AX / DAPI (Arbitrary Units)
10
5
0
***
***
***
***
**
**
**
**
i
ii
iii
iv
v
vi
vii
viii
Cells P P P P C B C B
IR - - + + - - + +
17-AAG - + - + - - - -

B

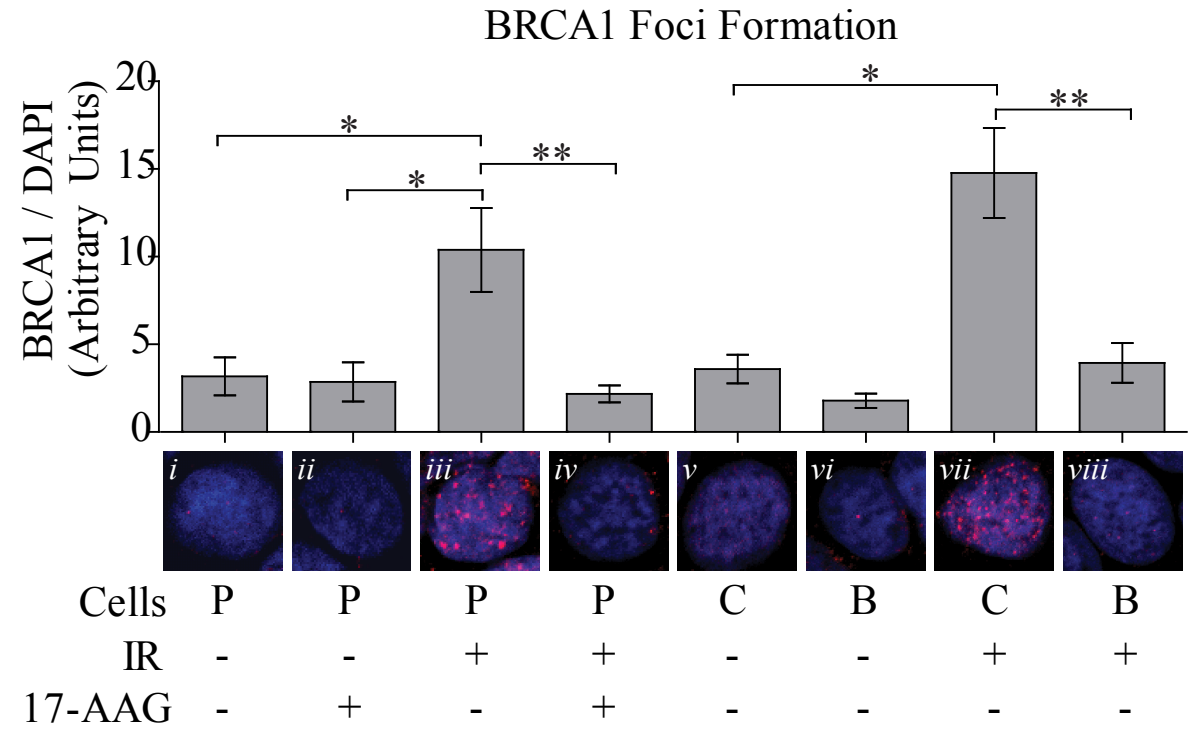
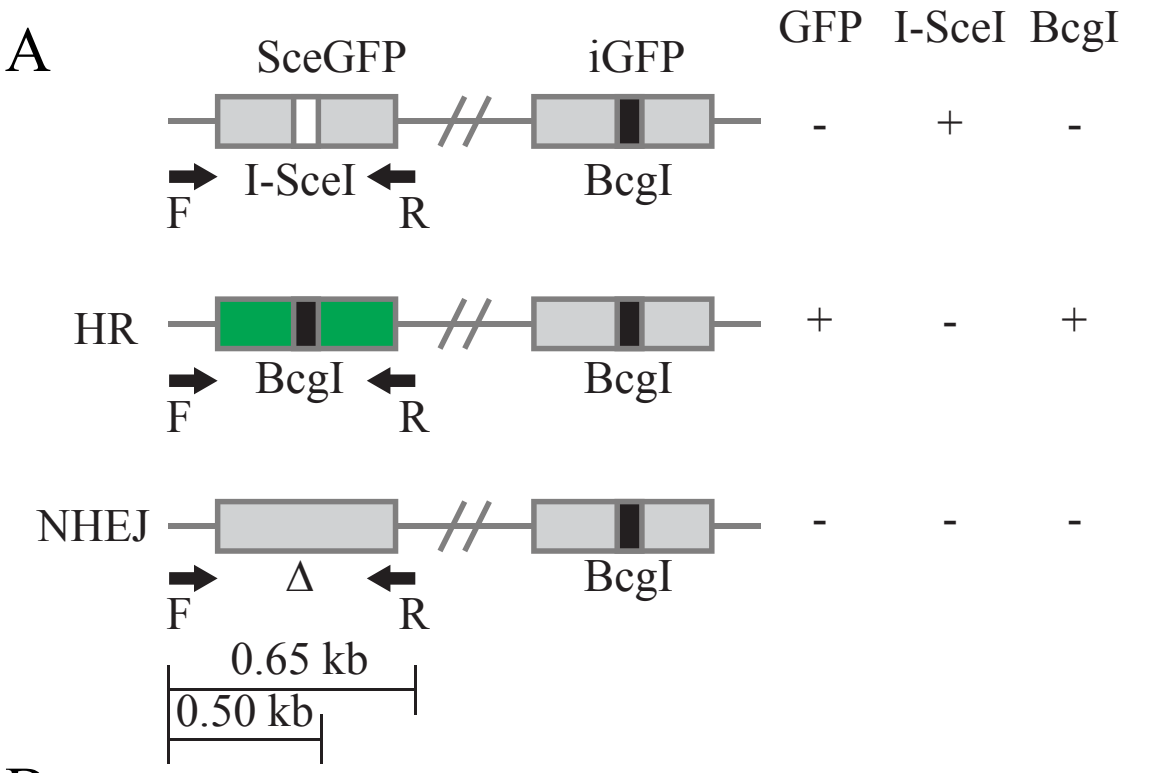

BRCA1 Foci Formation
BRCA1 / DAPI (Arbitrary Units)
20
15
10
5
0
*
*
**
*
**
i
ii
iii
iv
v
vi
vii
viii
Cells P P P P C B C B
IR - - + + - - + +
17-AAG - + - + - - - -

Figure 10. 17-AAG augments double-strand break induction, but abolishes BRCA1 localization ionizing radiation-induced damage. MCF7 cells were treated with DMSO or 250 nM 17-AAG for 24 hours. Cells were exposed to 0 or 10 Gy of ionizing radiation (IR) and then were fixed and immunostained for γH2AX (A) or BRCA1 (B) four hours post IR. Graphs represent the fluorescence intensity of the γH2AX or BRCA1 channel normalized to the DAPI channel in five random fields from one representative experiment. "Cells" designations; P = Parental MCF7, C = Control shRNA, B = BRCA1 shRNA1. Error bars represent SEM; *$p<0.05$, **$p<0.01$, ***$p<0.001$, student's t-test.

(Arlander et al., 2003; Dote et al., 2006; Noguchi et al., 2006; Oda et al., 2007), BRCA1 is required for the recruitment and function of all of these components (Bhattacharyya et al., 2000; Chen et al., 2008; D'Andrea and Grompe, 2003; Folias et al., 2002; Yarden et al., 2002; Zhang et al., 2009). Thus, our data suggest that loss of BRCA1 is a key upstream event leading to failure of DSB repair processes following inhibition of HSP90.

Inhibition of HSP90 Impairs Both Homologous Recombination and Non-Homologous End Joining

To functionally assess DSB repair capacity following treatment with 17-AAG, we employed a DSB repair reporter system (Figure 11A) (Nakanishi et al., 2005). To preclude the possibility that 17-AAG treatment would interfere with expression of I-SceI (and thus not permit valid interrogation of DSB repair using this assay), we evaluated the effect of 17-AAG treatment on expression of HA-tagged I-SceI. No difference in I-SceI stability was noted in the presence or absence of 17-AAG, excluding the possibility that observed differences in HR efficiency are due to impaired induction of DSBs in 17-AAG treated cells (Figure 11B). We used HeLa cells for this assay as they demonstrate more robust capacity for HR than do MCF7 cells, express higher levels of BRCA1, and have been used to examine BRCA1-associated defects in HR (Kachhap et al., 2001; Ransburgh et al., 2010). HeLa cells which had been stably selected to contain the DR-GFP reporter construct were electroporated with a control vector (Figure 12*i* and *ii*) or a vector encoding I-SceI (Figure 12*iii* and *iv*) and were immediately plated into media containing DMSO (Figure 12*i* and *iii*) or 250 nM 17-AAG (Figure 12*ii* and *iv*). Approximately 14.8% of the vehicle-treated cells expressing I-SceI exhibited HR (Figure 12*iii*), while 0.4% of those treated with 17-AAG completed HR (Figure 12*iv*). To confirm reduced HR and to assess the


A
SceGFP
iGFP
GFP
I-SceI
BcgI
-
+
-
I-SceI
BcgI
F
R
HR
+
-
+
BcgI
BcgI
F
R
NHEJ
-
-
-
Δ
BcgI
F
R
0.65 kb
0.50 kb

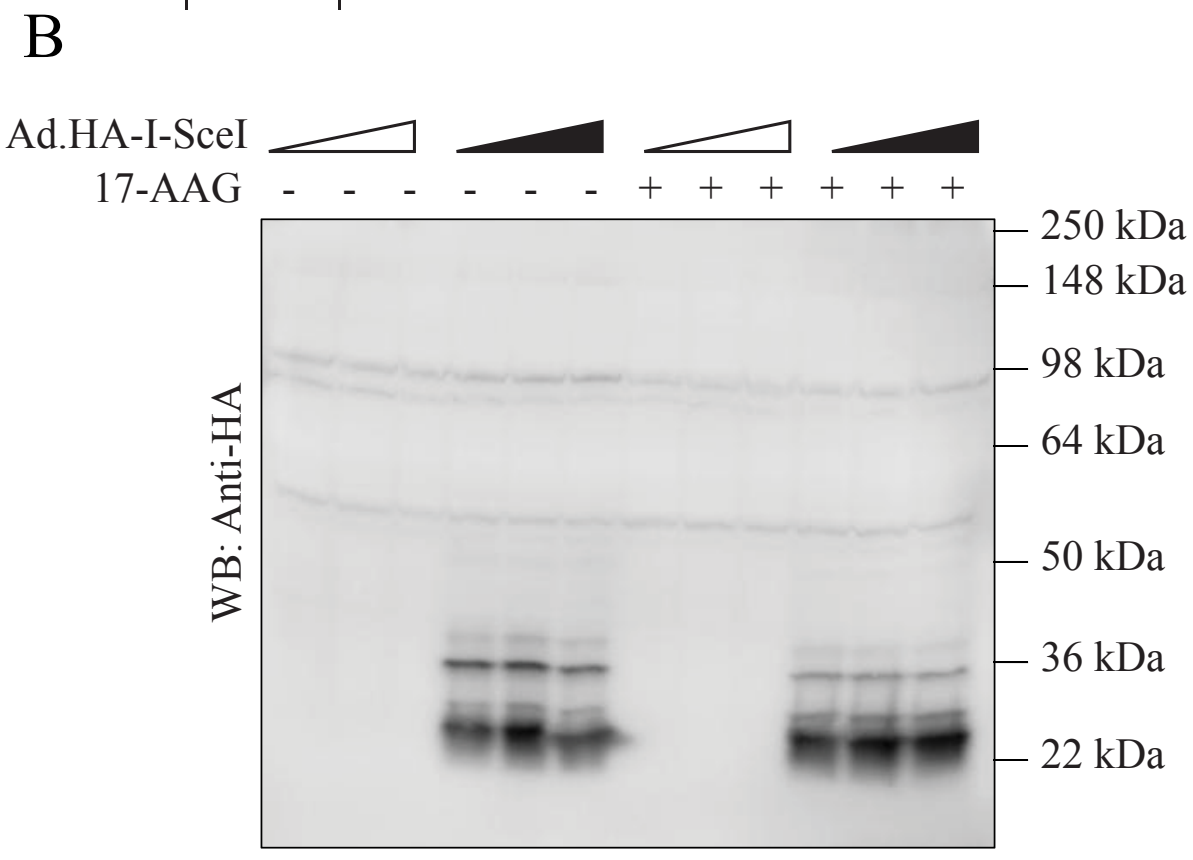
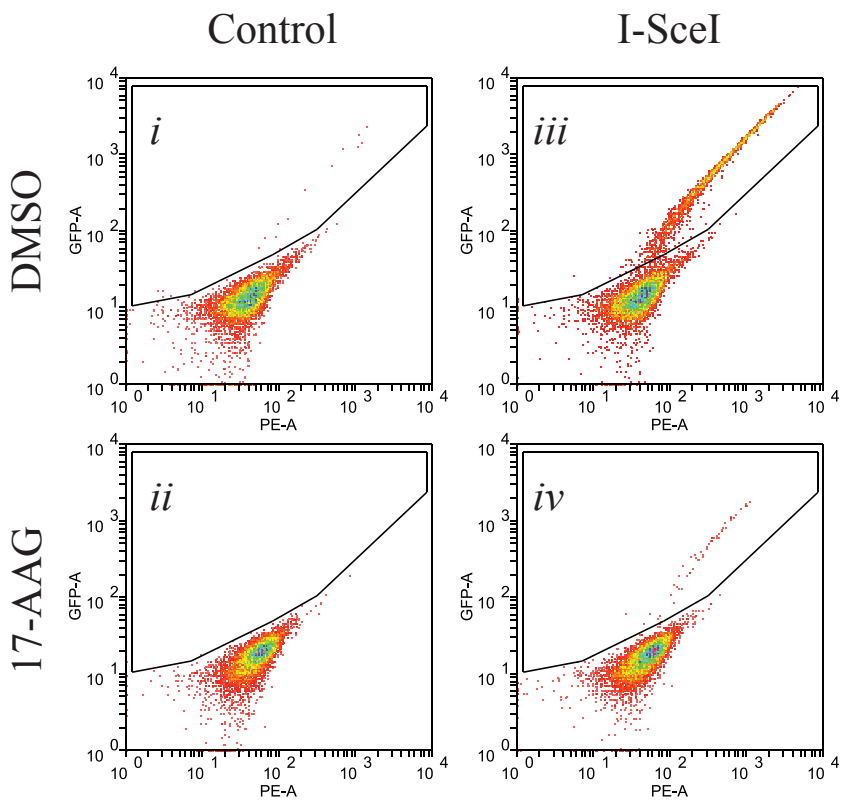

B
Ad.HA-I-SceI
17-AAG
- - - - - - + + + + + +
WB: Anti-HA
250 kDa
148 kDa
98 kDa
64 kDa
50 kDa
36 kDa
22 kDa

Figure 11. HR/NHEJ functional reporter assay. (A) Schematic of the integrated DR-GFP reporter before and after HR or NHEJ. (B) HeLa cells were infected with an adenovirus encoding HA-tagged I-SceI (closed triangles) or an empty adenovirus (open triangles) and plated as descrbed for the HR/NHEJ assay. Total protein was isolated, electrophoresed and probed with an anti-HA antibody.


Control
I-SceI
DMSO
17-AAG
i
ii
iii
iv
GFP-A
PE-A

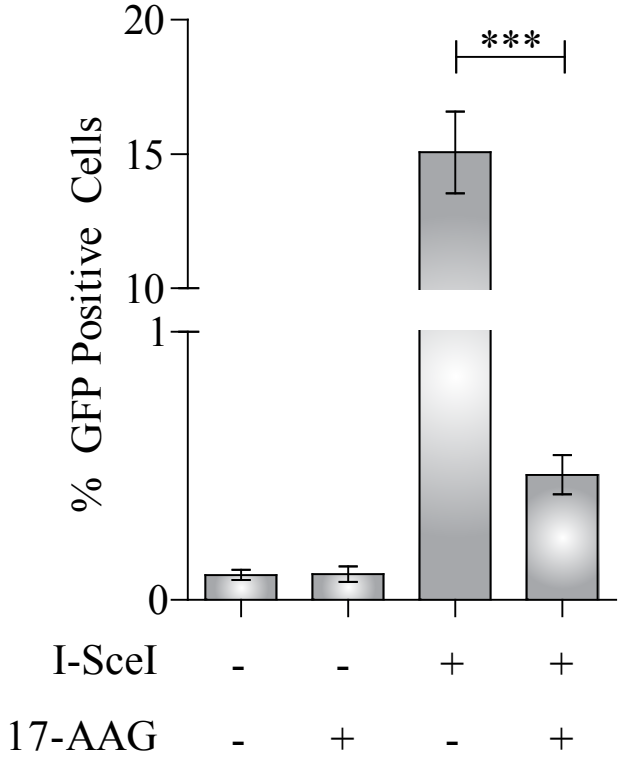

% GFP Positive Cells
20
15
10
1
0
***
I-SceI - - + +
17-AAG - + - +

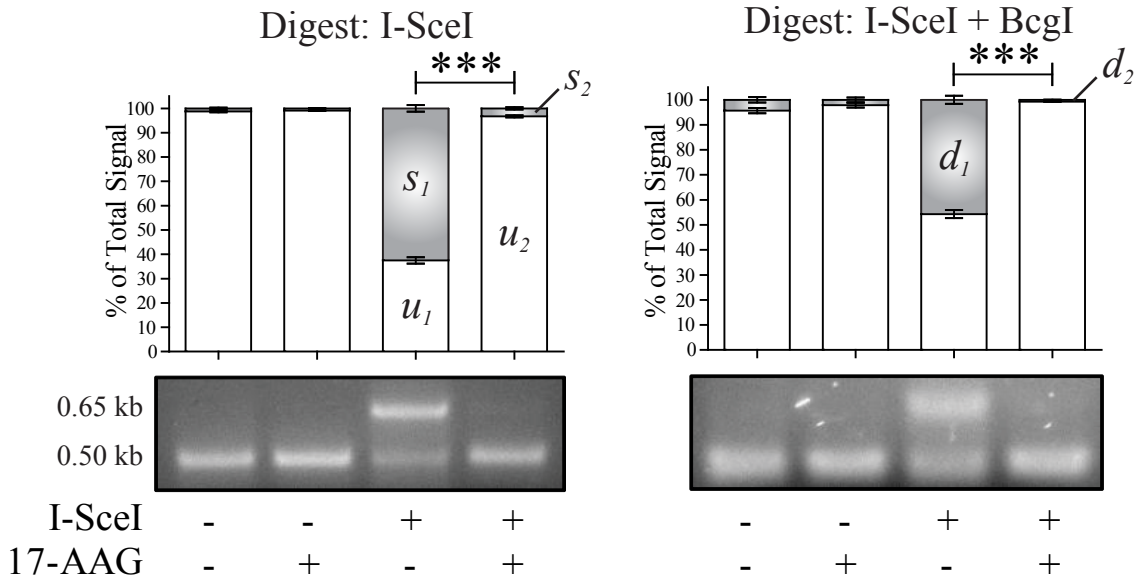
Figure 12. Inhibition of HSP90 impairs homologous recombination. HeLa cells stably expressing the DR-GFP reporter were electroporated with either control empty vector or a vector expressing I-SceI and were immediately plated into either DMSO or 250 nM 17-AAG for 24 hours. Media was replaced after 24 hours (no drug included) and cells were incubated for an additional 24 hours before flow cytometric analysis. Gated cells express GFP, indicating successful HR. Graph depicts number of GFP+ cells in three independent experiments. n=3. Error bars represent SEM; *$p<0.05$, **$p<0.01$, ***$p<0.001$, student's t test.

contribution of NHEJ to DSB repair following I-SceI expression, we performed PCR amplification and enzymatic digestion of repair products from genomic DNA isolated from the reporter HeLa cells. Amplification of the region indicated by arrows in Figure 11A will generate a 0.65 kb fragment. Total repair product (i.e., repaired by either HR or NHEJ) can be visualized as the relative amount of PCR product that remains uncut following digestion with I-SceI (Figure 13A, "s_1"). Vehicle-treated HeLa cells expressing I-SceI demonstrated robust DSB repair (62.5%), while those treated with 17-AAG had profoundly lower total repair product (3.3%) (Figure 13A, compare "*s1*" and "*s2*"). Subsequent digestion of the I-SceI-cleaved product with BcgI will delineate the individual contributions of HR and NHEJ to total DSB repair. From these data, it was calculated that in vehicle-treated cells, approximately 75% of total repair product was repaired by NHEJ, while 25% was repaired by HR (Figure 13B, left). While 17-AAG significantly interfered with both HR and NHEJ, the distribution of repair pathway choice was only marginally skewed, with 86% and 14% repaired by NHEJ and HR, respectively (Figure 13B, right). These values are consistent with the flow cytometric assay of HR, as 25% of 62.5%, and 14% of 3.3% for DMSO and 17-AAG treated cells, respectively, would generate expected GFP+ frequencies of 15.6% and 0.5% (compared to the observed 14.8% and 0.4%). These data provide functional support of our finding that 17-AAG impairs BRCA1 expression and function and supports previous studies suggesting that HSP90 is required for both HR and NHEJ (Dote et al., 2006; Noguchi et al., 2006; Oda et al., 2007).

Discussion

In this chapter, we have demonstrated that the HSP90 chaperone is required for maintenance of BRCA1 expression and function. To our knowledge, this study is the first to document a

A


Digest: I-SceI
Digest: I-SceI + BcgI
***
***
s_2
d_2
% of Total Signal
s_1
u_2
u_1
d_1
0.65 kb
0.50 kb
I-SceI
17-AAG

B

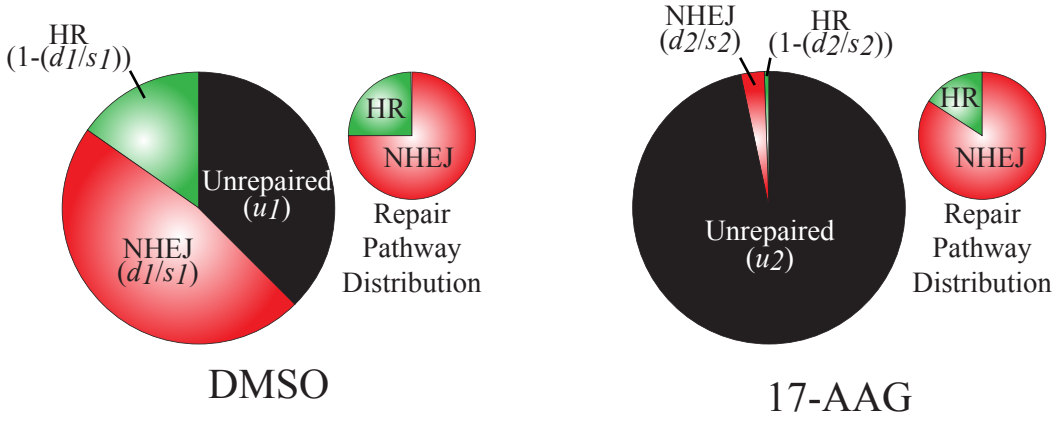

HR
(1-(d1/s1))
Unrepaired
(u1)
NHEJ
(d1/s1)
HR
NHEJ
Repair
Pathway
Distribution
DMSO
NHEJ
(d2/s2)
HR
(1-(d2/s2))
Unrepaired
(u2)
HR
NHEJ
Repair
Pathway
Distribution
17-AAG

Figure 13. Inhibition of HSP90 impairs non-homologous end joining. HeLa cells stably expressing the DR-GFP reporter were electroporated with either control empty vector or a vector expressing I-SceI and were immediately plated into either DMSO or 250 nM 17-AAG for 24 hours. Media was replaced after 24 hours (no drug included) and cells were incubated for an additional 24 hours. The region denoted by arrows in Figure 11A was PCR amplified from genomic DNA and subjected to I-SceI and BcgI digestion. (A) I-SceI and I-SceI+BcgI digested PCR products. Grey bars in graph represent uncut (0.65 kb) fragment. (B) Summary of total repair capacity and repair pathway distribution in I-SceI transfected cells. Small graphs represent distribution of total repair product (HR+NHEJ). n=3. Error bars represent SEM; *$p<0.05$, **$p<0.01$, ***$p<0.001$, student's t test.

functional link between HSP90 and BRCA1. Specifically, we demonstrated that pharmacologic inhibition of HSP90 using the clinically-relevant inhibitor 17-AAG results in destabilization and proteasomal degradation of BRCA1. In an attempt to test the alternative hypothesis that HSP90 regulates BRCA1 stability indirectly, we evaluated the expression of BARD1, a constitutive BRCA1 binding partner that is known to be critical for BRCA1 stability (Joukov et al., 2001). BARD1 downregulation was significantly delayed compared to BRCA1 degradation and is likely due to destabilization of BRCA1 rather than a direct consequence of HSP90 inhibition.

Prior to our study, a single report suggested a link between BRCA1 and the heat shock response (Xian Ma et al., 2003). This report showed that BRCA1 plays a protective role in heat toxicity and that thermal stress induces BRCA1 degradation. While this study observed profound destabilization of BRCA1 in breast and prostate cancer cells in response to incubation at 42°C, they were unable to rescue BRCA1 protein levels by inhibiting any of the well-characterized pathways that mediate protein degradation, including the ubiquitin-proteasome pathway. Thus, our results, which document that the ubiquitin-proteasome pathway is responsible for BRCA1 degradation suggests that the mechanism of BRCA1 degradation in response to heat shock is quite distinct from the mechanism of BRCA1 degradation in response to heat shock protein 90 inhibitors.

Our study also documents that HSP90 inhibitors prevent the localization of BRCA1 to IRIF. While previous studies have described failed homology-directed repair of DSBs after inhibition of HSP90, these studies have focused on the effects of HSP90 inhibitors on BRCA2, RAD51, FANCA, CHK1, and the MRE11/RAD50/NBS1 (MRN) complex (Arlander et al., 2003; Dote et

al., 2006; Noguchi et al., 2006; Oda et al., 2007). Importantly, the function of each of these molecules is dependent upon BRCA1. Specifically, evidence suggests that BRCA2, RAD51, and the MRN complex fail to assemble in the absence of BRCA1 (Bhattacharyya et al., 2000; Zhang et al., 2009; Zhong et al., 1999). Furthermore, BRCA1 is necessary for activation of CHK1 kinase which is critical in inducing G_2/M arrest following DNA damage and functioning in concert to facilitate the monoubiquitination of FANCD2 (Guervilly et al., 2008; Yarden et al., 2002). Thus, of the HR/ICLR repair proteins that have been identified to be sensitive to HSP90 inhibitors, BRCA1 appears to be the most upstream.

As modeled in Figure 14, certain types of DNA damage caused by chemical or physical agents induce BRCA1 expression and localization to sites of DSBs, ultimately resulting in DNA repair by either HR/ICLR or NHEJ. While this sequence of events is protective and desirable in normal tissue, hyperactivity of these BRCA1-dependent DNA damage repair complexes can confer resistance to cancer therapeutics, thus the therapeutic benefit of traditional genotoxic agents is likely stunted by the action(s) of BRCA1 and its associated proteins. Agents which inhibit the BRCA1-dependent DNA damage repair machinery may find success in enhancing or restoring sensitivity to therapeutic agents in both the adjuvant and salvage therapy settings.

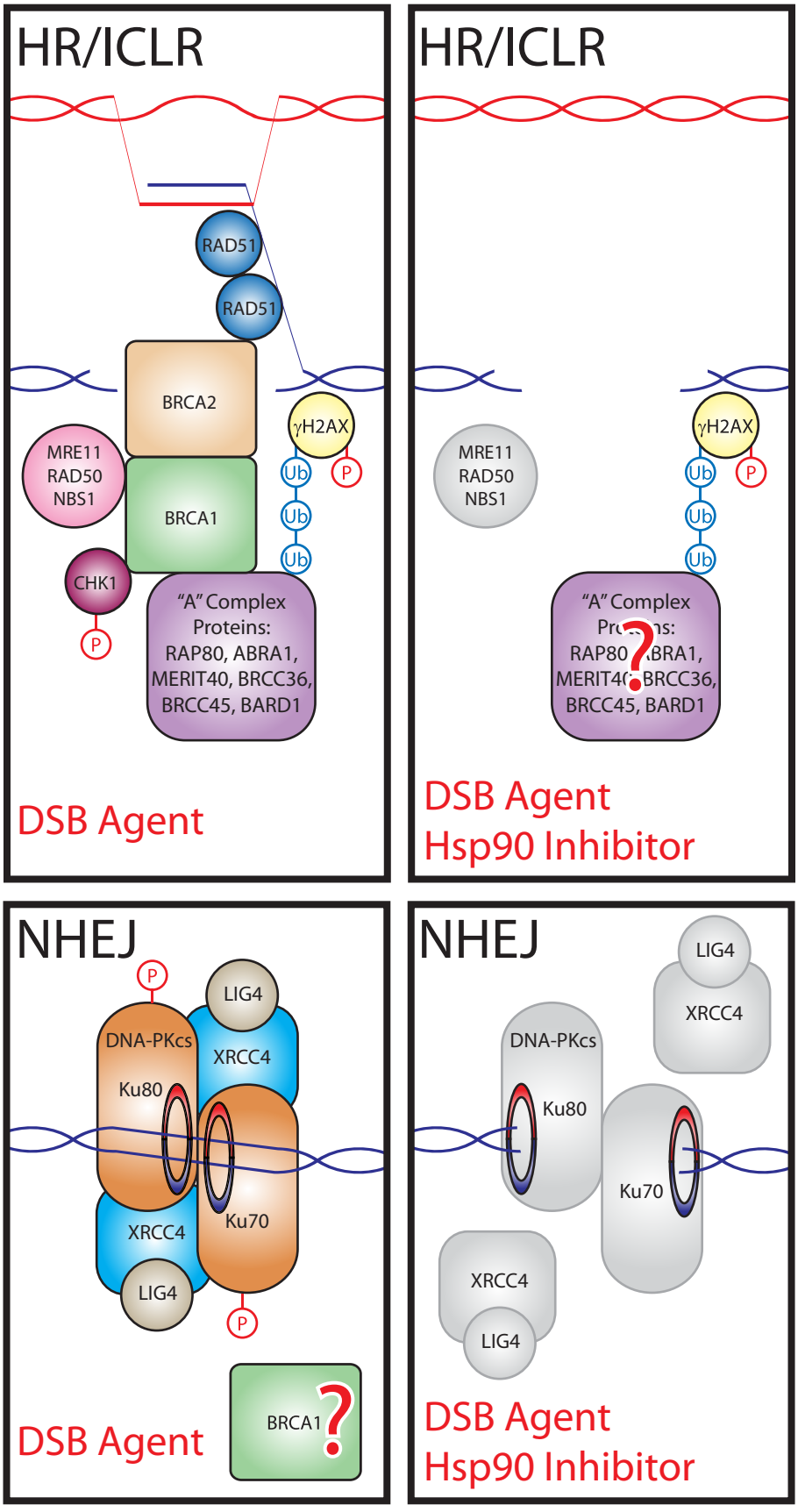

HR/ICLR
RAD51
RAD51
BRCA2
γH2AX
MRE11
RAD50
NBS1
BRCA1
Ub
Ub
Ub
P
CHK1
P
"A" Complex
Proteins:
RAP80, ABRA1,
MERIT40, BRCC36,
BRCC45, BARD1
DSB Agent
HR/ICLR
MRE11
RAD50
NBS1
γH2AX
Ub
Ub
Ub
P
"A" Complex
Pro ns:
RAP80 BRA1,
MERIT4 BRCC36,
BRCC45, BARD1
?
DSB Agent
Hsp90 Inhibitor
NHEJ
P
LIG4
DNA-PKcs
XRCC4
Ku80
Ku70
XRCC4
LIG4
P
DSB Agent
BRCA1
?
NHEJ
LIG4
XRCC4
DNA-PKcs
Ku80
Ku70
XRCC4
LIG4
DSB Agent
Hsp90 Inhibitor

Figure 14. Model for regulation of BRCA1-dependent DNA damage repair by HSP90. HR/ICLR: Upon exposure to ionizing radiation or other agents which induce DSBs, gH2AX becomes phosphorylated and ubiquitinated. This histone modification serves as a docking site for components of the BRCA1 "A" complex, including RAP80, ABRA1, MERIT40, BRCC36, BRCC45, and BARD1. BRCA1 assembly allows the recruitment of the MRE11/RAD50/NBS1 (MRN) complex, the Fanconi Anemia complex including BRCA2 and RAD51, and phosphorylation of CHK1, facilitating error-free repair and activation of the G2/M checkpoint. Treatment with 17-AAG does not impair, and in fact may augment the early steps of double strand break signaling (i.e., gH2AX phosphorylation) but interferes with BRCA1 assembly at sites of DSBs. This would be expected to lead to a defect in the assembly of downstream components of the DSB repair machinery including MRN and BRCA2/RAD51. Whether components of the "A" complex are regulated by HSP90 remains unknown. NHEJ: The Ku70/Ku80 heterodimer associates with the free ends of the DSB and recruits DNA-PKcs. Activation of DNA-PKcs is mediated by phosphorylation, which then recruits XRCC4 and DNA Ligase 4. The role(s) of BRCA1 in NHEJ remain poorly described. Grey boxes indicate failed activation or localization in the presence of HSP90 inhibitors.

Chapter III: BRCA1 Regulates 17-AAG-Induced Radio- and Chemosensitization, G2/M Checkpoint Activation, Replication Stress Response and Apoptosis

Introduction

BRCA1 is a critical determinant of homology directed repair of double-strand DNA lesions arising from ionizing radiation (IR) and interstrand crosslinks (ICLs) and also prevents collapse and promotes error-free bypass of stalled replication forks by sister chromatid exchange. Though BRCA1 is a tumor suppressor and is critical for genomic integrity, *in vitro*, *in vivo* and human clinical studies have shown that tumor cells expressing high levels of BRCA1 are resistant to IR and several classes of chemotherapeutic agents and that ablation of BRCA1 expression in resistant cell lines can restore sensitivity to these agents (Abbott et al., 1999; Scata and El-Deiry, 2007). Consistent with these findings, cancers arising in *BRCA1* mutation carriers are profoundly sensitive to platinum-based therapies while high BRCA1 mRNA expression in sporadic cancers is a biomarker for poor response to these same agents (Bhattacharyya et al., 2000; Husain et al., 1998; Silver et al.). Since BRCA1 is critical for resolving double-strand breaks (DSBs) and ICLs, cells with exaggerated BRCA1 expression are likely selected for during treatment with genotoxic agents both *in vitro* and *in vivo*. While BRCA1- and BRCA2-deficient cancers are profoundly sensitive to platinum-based therapies and PARP inhibitors, recent studies of *BRCA1*/*2*-associated human ovarian cancers have revealed that therapy-induced secondary mutations which restore the *BRCA1* or *BRCA2* reading frames occur with significant frequency and are directly responsible for resistance to platinum-based therapies and PARP inhibitors (Ashworth, 2008; Swisher et al., 2008). Thus, targeting BRCA1 and/or its associated protein complexes in cancer chemo- and radiotherapy may induce hypersensitivity to agents which induce DSBs and prevent the development or recurrence of resistant disease (Chen et al., 2006).

In this chapter, we investigated the ability of the HSP90 inhibitor 17-AAG to enhance or restore sensitivity to ionizing radiation and carboplatin in several models. We demonstrate that lentiviral knockdown of BRCA1 or inhibition of HSP90 with 17-AAG leads to hypersensitivity to ionizing radiation in MCF7 breast cancer cells, and that loss of BRCA1 is epistatic to the ability of 17-AAG to further sensitize cells to radiation. Moreover, we show that genetic complementation of wild-type *BRCA1* into *BRCA1*-mutant ovarian and breast cancer cells induces resistance to carboplatin, and that 17-AAG can reverse this resistance. We extend these studies to show that 17-AAG can also sensitize sporadic platinum-resistant ovarian cancer cells. In summary, we demonstrate the therapeutic utility of ablating BRCA1 and identify a novel approach to reversing platinum and radiation resistance in both breast and ovarian cancer.

Materials and Methods

Cell Culture

A2780, CP70 and C30 cells were provided by Andrew Godwin (University of Kansas Medical Center). HCC1937 and HCC1937^{BRCA1} cells were a gift from Junjie Chen (MD Anderson Cancer Center). All five lines were grown in RPMI-1640 supplemented with 10% FBS and penicillin/streptomycin. All other cell lines were obtained from ATCC. Culture of all cell lines was performed per ATCC recommendations.

Lentivirus Generation and Infection

The Expression Arrest™ GIPZ lentiviral shRNAmiR library targeting BRCA1 and control GIPZ vector were purchased from OpenBiosystems (ThermoFisher Scientific, Waltham, MA). Lentiviruses were packaged in HEK293T cells using pRC-CMV-RaII, pHDM-tat16, pHDM-

HG-PM2, and pHDM-VSVG packaging constructs. Supernatants were ultracentrifuged and infectious particles were titered by flow cytometry according to the equation $= \frac{P \cdot N}{D \cdot V}$, where T equals titer in transducing units (TU) per mL, P equals the percentage of GFP^+ cells, N equals the number of cells at the time of transduction, D equals the dilution of virus, and V equals the volume of viral inoculum in mL. MCF7 cells were infected at a multiplicity of infection (MOI) of 0.2 according to the equation $V = \frac{C \cdot MOI}{T}$, where V equals the volume of stock virus required in mL, C equals the number of cells being infected, MOI equals the desired multiplicity of infection, and T equals titer (in TU/mL) calculated as described above. Cells were selected for two weeks in 2 µg/mL puromycin and were maintained in selective media for the duration of all experiments.

Drug Treatment

17-AAG was purchased from InvivoGen and dissolved in DMSO. Carboplatin was purchased from Sigma-Aldrich and was dissolved in PBS.

Irradiation

Cells were irradiated using a ^{137}Cs source irradiator at a dose rate of 4.97 $Gy \cdot min^{-1}$.

Antibodies

Mouse anti-Actin (#MAB1501, Upstate/Millipore), mouse anti-BRCA1 (Ab1) (#OP92, Calbiochem/EMD Biosciences), mouse anti-HSP70 (#610608, BD Biosciences), mouse anti-HSP27 (#2402, Cell Signaling Technology), rabbit anti-HSF1 (#4356, Cell Signaling Technology), mouse anti-CHK1 (#2360, Cell Signaling Technology), rabbit anti-$pCHK1^{S345}$

(#2341, Cell Signaling Technology), rabbit anti-cyclin B1 (#sc-752, Santa Cruz Biotechnology), rabbit anti-H3 (#9715, Cell Signaling Technology), rabbit anti-$pH3^{S10}$ (#9701, Cell Signaling Technology), Alexa®-488 anti-$pH3^{S28}$ (#558610, BD Biosciences), anti-rabbit HRP-linked (#7074, Cell Signaling Technology) and anti-mouse HRP-linked (#7076, Cell Signaling Technology).

Western Blot

Protein was harvested using a modified RIPA buffer (50 mM Tris base, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM NaF, 5 mM Na_3VO_4, plus 1X protease inhibitor cocktail (Sigma-Aldrich)), quantified using the D_C protein assay (Bio-Rad), electrophoresed on 8% or 4-12% tris-glycine gels (Invitrogen) and then transferred onto PVDF membranes. All blocking steps and antibody-dilutions were in 3% non-fat dairy milk in PBS + 0.1% Tween-20.

Clonogenicity Assay

MCF7 cells infected with control or BRCA1-targeting lentivirus were pretreated for 48 hours with 10 nM 17-AAG or DMSO control. Cells were trypsinized, counted, and 3,000 cells were plated in 60 mm dishes with fresh 17-AAG or DMSO. After resting overnight, plates were radiated with 0, 1, 2, 4, or 6 Gy and were then incubated for an additional 10 days. Plates were fixed with 100% methanol, stained with 1% crystal violet, and colonies were imaged and counted using the UVP BioImaging System and ImageJ, respectively.

MTS Assay

Cells were plated at $3x10^5$ (HCC1937 and UWB1.289) or $5x10^3$ (MCF7) cells per well in a 96-well plate and were treated for 4 (MCF7) or 5 (HCC1937 and UWB1.289) days with indicated doses of 17-AAG or carboplatin. Assay was performed using the CellTiter 96® AQ_{ueous} MTS kit (Promega) according to the manufacturer's instructions.

Cell Cycle, DNA Synthesis and Apoptosis Assays

HCC1937 and $HCC1937^{BRCA1}$ cells were treated with 250 nM 17-AAG or DMSO for indicated times. For cell cycle analysis, cells were trypsinized and resuspended in 1 mL of 0.9% NaCl, and vortexed gently during dropwise addition of 2.5 mL 90% cold ethanol. After 30 minutes of fixation on ice, cells were stained with 50 μg/mL propodium iodide and treated with RNase A for 30 minutes at room temperature. For studies examining mitotic entry, cell cycle staining was completed as described, with the addition of Alexa®-488 anti-$pH3^{S28}$ (#558610, BD Biosciences) staining step per the manufacturer's instructions. DNA synthesis and TUNEL staining was performed using the Click-iT® EdU cell proliferation assay (Invitrogen) and the Click-iT TUNEL assay (Invitrogen) as described by the manufacturer. Annexin staining assay was done with V450 Annexin V (BD Biosciences, #560506) per manufacturer supplied protocol. All experiments were analyzed on LSR II flow cytometer (BD Biosciences) except for the TUNEL assay which was analyzed on a Celigo adherent cell cytometer (Cyntellect).

Results

17-AAG Regulates the Stability of Both Wild-Type and Mutant Forms of BRCA1

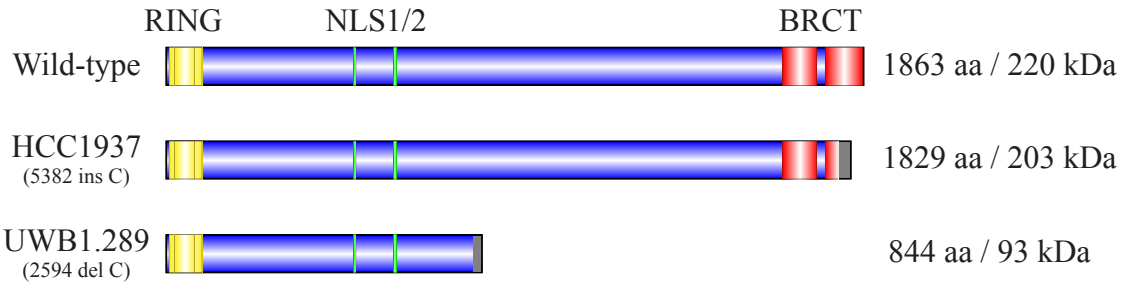
Because mutation-associated resistance to platinum-based chemotherapeutics and PARP inhibitors arises from intragenic alterations in mutant *BRCA1/2* alleles, we assessed the ability of

17-AAG to destabilize mutant forms of BRCA1 protein in HCC1937 breast cancer cells (homozygous *BRCA1* 5382insC) and UWB1.289 ovarian cancer cells (homozygous *BRCA1* 2594 del C) (Figure 1A). 17-AAG treatment resulted in downregulation of both BRCA1 mutants (Figure 1B), although to a less dramatic extent than in the *BRCA1* wild-type cells examined previously (Chapter II Figures 1A-B and 6A). To determine whether the kinetics of wild-type and mutant BRCA1 loss differed in isogenic cell lines, we evaluated the response of HCC1937 cells as well as a wild-type *BRCA1*-complemented clone (designated HCC1937^{BRCA1}) to 250 nM 17-AAG for 0-5 days. Wild-type and mutant BRCA1 were lost at similar rates, with a nadir of approximately 40% at day 3 in both cell lines (Figure 2A-B).

BRCA1 Expression Regulates Sensitivity to 17-AAG and Mediates HSP90 Inhibitor-Induced Sensitivity to Ionizing Radiation

Since BRCA1 is a central scaffolding protein for assembling the HR machinery, loss of BRCA1 should eliminate synergy between 17-AAG and agents which induce HR-mediated repair. To address this, we generated MCF7 cells with reduced expression of BRCA1 via lentiviral shRNAs. Three populations of MCF7 cells were selected with variable levels of BRCA1 expression, though HSP90 expression was unaffected (Figure 3A). Given our previous finding that 17-AAG induces or augments γH2AX phosphorylation (i.e., enhances accumulation of DSBs) (Chapter II Figure 10), we hypothesized that BRCA1-deficient cells would be intrinsically sensitive to 17-AAG. MCF7 cells with variable expression of BRCA1 were treated with increasing doses of 17-AAG for four days and subjected to MTS assay. The ability to proliferate in the presence of 17-AAG closely parallels the level of BRCA1 expression (Figure 3A-B). The IC_{50} values of shRNA1, shRNA2, shRNA3 and control populations were 137 nM,

A


RING
NLS1/2
BRCT
Wild-type
1863 aa / 220 kDa
HCC1937
(5382 ins C)
1829 aa / 203 kDa
UWB1.289
(2594 del C)
844 aa / 93 kDa

B

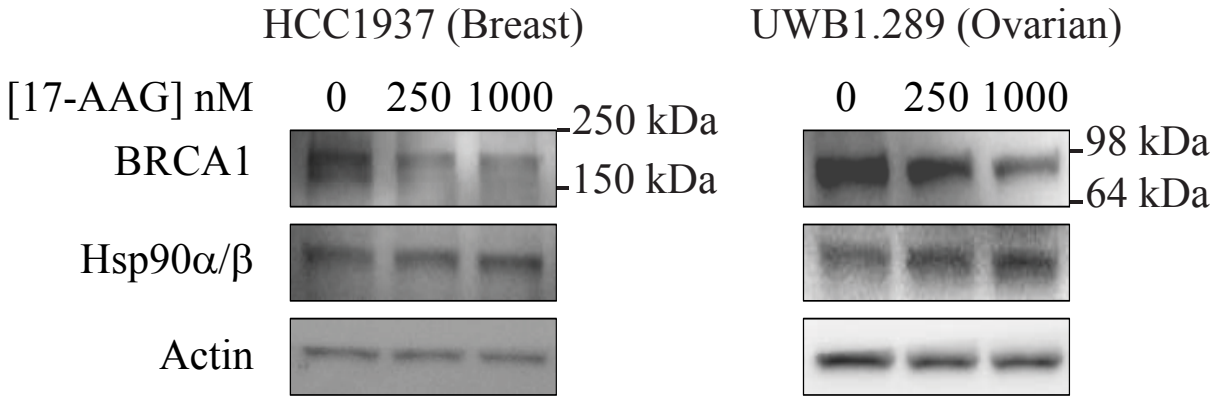
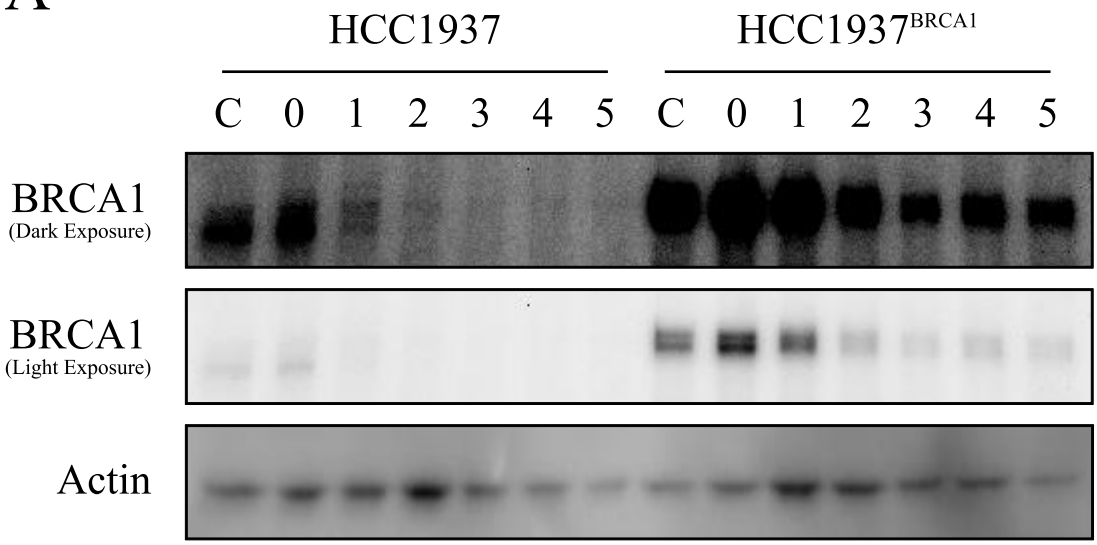

HCC1937 (Breast)
UWB1.289 (Ovarian)
[17-AAG] nM
0 250 1000
0 250 1000
BRCA1
250 kDa
150 kDa
98 kDa
64 kDa
Hsp90α/β
Actin

Figure 1. Inhibition of HSP90 destabilizes mutant forms of BRCA1. (A) Diagrammatic representation of the predicted mutant BRCA1 protein in HCC1937 and UWB1.289 cells. (B) Western blots of HCC1937 and UWB1.289 cells treated with indicated concentrations of 17-AAG for eight hours.

A


HCC1937
HCC1937BRCA1
C 0 1 2 3 4 5 C 0 1 2 3 4 5
BRCA1
(Dark Exposure)
BRCA1
(Light Exposure)
Actin

B

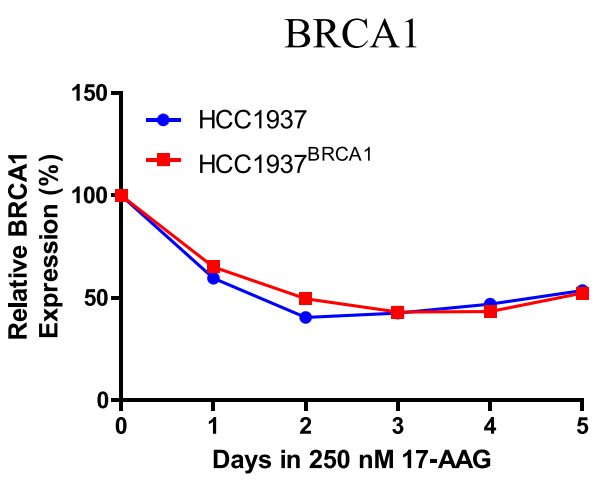
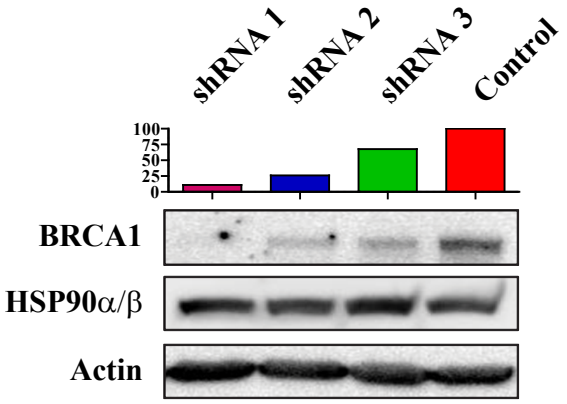
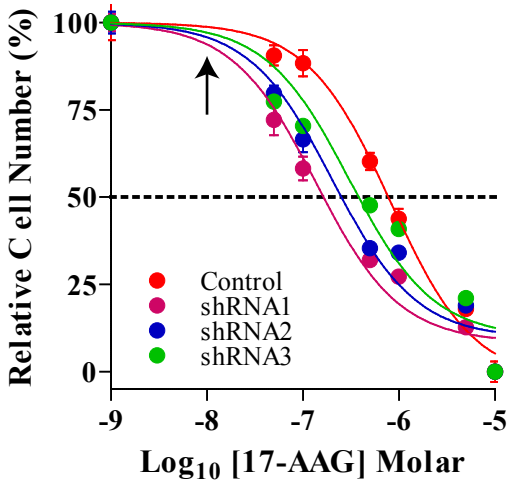

BRCA1
HCC1937
HCC1937BRCA1
Relative BRCA1 Expression (%)
150
100
50
0
0 1 2 3 4 5
Days in 250 nM 17-AAG

Figure 2. 17-AAG destabilizes wild-type and mutant BRCA1 with similar kinetics. (A) HCC1937 and HCC1937^{BRCA1} cells were grown in 250 nM 17-AAG for the indicated number of days (C indicates no treatment, '0' indicates vehicle control). Total protein was isolated, electrophoresed and probed with the indicated antibodies. (B) Graphical representation of BRCA1 protein levels in HCC1937 and HCC1937^{BRCA1} cells following 17-AAG treatment. Density of BRCA1 band was normalized to corresponding density of actin band, and then all timepoints were normalized to the vehicle control lane.

A


shRNA 1
shRNA 2
shRNA 3
Control
100
75
50
25
0
BRCA1
HSP90α/β
Actin

B


Relative C ell Number (%)
100
75
50
25
0
-9
-8
-7
-6
-5
Log10 [17-AAG] Molar
Control
shRNA1
shRNA2
shRNA3

C

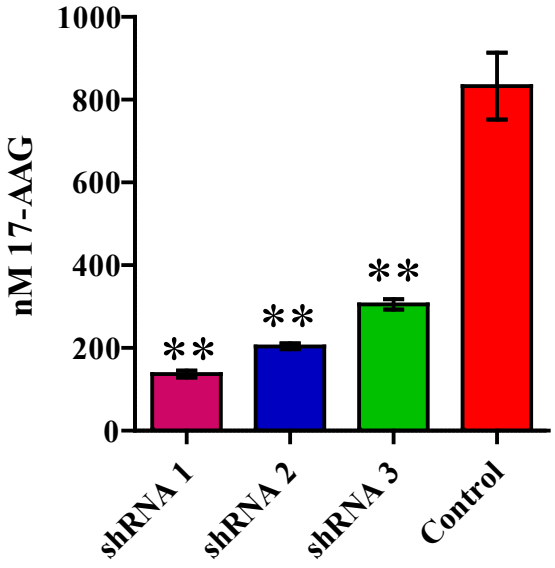

nM 17-AAG
1000
800
600
400
200
0
**
**
**
shRNA 1
shRNA 2
shRNA 3
Control

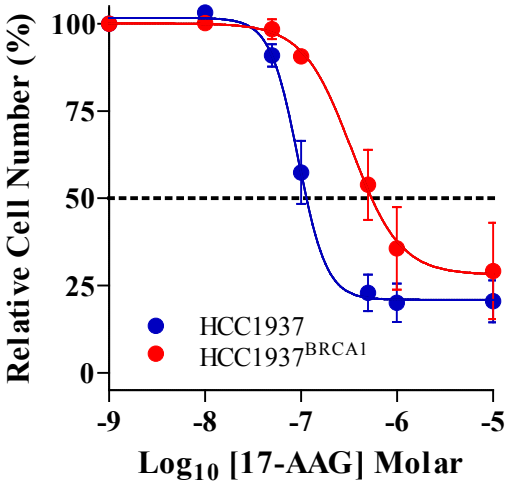
Figure 3. Lentiviral ablation of BRCA1 increases sensitivity to 17-AAG in MCF7 cells. (A) Western blots of MCF7 cells infected with lentiviruses expressing control or BRCA1-targeting shRNAs. The graph above the BRCA1 blot indicates the ratio of BRCA1 band intensity of each BRCA1-targeting lentivirus compared to the control lentivirus, normalized to actin loading control. (B) MTS assay of BRCA1-shRNA lentivirus infected MCF7 cells treated with increasing concentrations of 17-AAG. (C) IC_{50} values based on data presented in panel B. Error bars represent SEM of four independent experiments. *$p<0.05$, **$p<0.01$, ***$p<0.001$ (student's t-test).

204 nM, 305 nM, and 832 nM, respectively (Figure 3C). We confirmed this finding in the *BRCA1* mutant breast cancer cell line HCC1937 and the *BRCA1* wild-type complemented clone $HCC1937^{BRCA1}$, where IC_{50} values were found to be 101 nM and 331 nM, respectively (Figure 4A-B). To address whether loss of BRCA1 is epistatic to the ability of 17-AAG to potentiate cells to ionizing radiation, the MCF7-shRNA2 and MCF7-control populations were treated with DMSO or 10 nM 17-AAG for two days and then were irradiated and assessed for clonogenic growth capacity (Figure 5A). This low dose and shorter duration of 17-AAG was selected because it effectively ablates BRCA1 expression (Chapter II Figure 1A-B) and has insignificant effects on the proliferation of both control and BRCA1-ablated MCF7 cells (Figure 5B and 3B (arrow)). 17-AAG treatment significantly potentiated MCF7-control cells to clinically-relevant doses of IR (Figure 5C-D). As expected, loss of BRCA1 alone also potentiated cells to IR (Figure 5C-D). Unexpectedly, 17-AAG treatment of BRCA1-deficient MCF7 cells revealed no synergy between 17-AAG and IR (Figure 5C-D). These data suggest that a major sensitizing effect of 17-AAG is due to loss of BRCA1. Our finding that loss of BRCA1 prevents any further potentiation has important implications and may provide further functional support for a role of BRCA1 in NHEJ.

17-AAG Exhibits Enhanced Synergy in Cells with Restored Wild-Type BRCA1 and can Re-Sensitize Platinum-Resistant Breast and Ovarian Cancer Cells

Overexpression of wild-type BRCA1 and frame-restoring intragenic mutations in *BRCA1* are associated with resistance to platinum-based chemotherapeutics and PARP inhibitors in ovarian cancer. To evaluate whether 17-AAG could mitigate repair-mediated resistance arising from BRCA1-dependent DNA damage repair, we evaluated the ability of 17-AAG to sensitize

A


Relative Cell Number (%)
100
75
50
25
0
-9
-8
-7
-6
-5
Log10 [17-AAG] Molar
HCC1937
HCC1937BRCA1

B

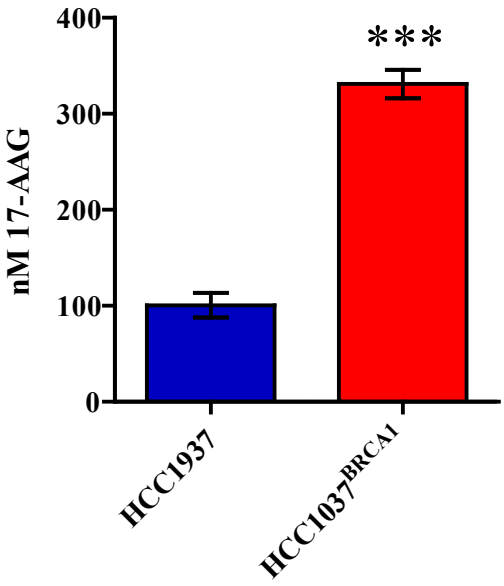
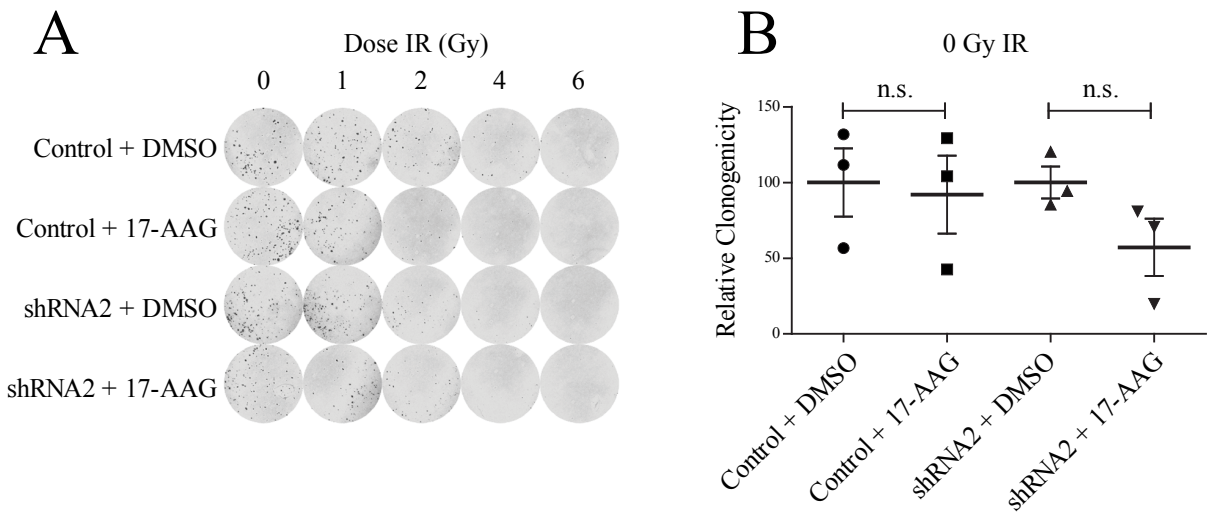
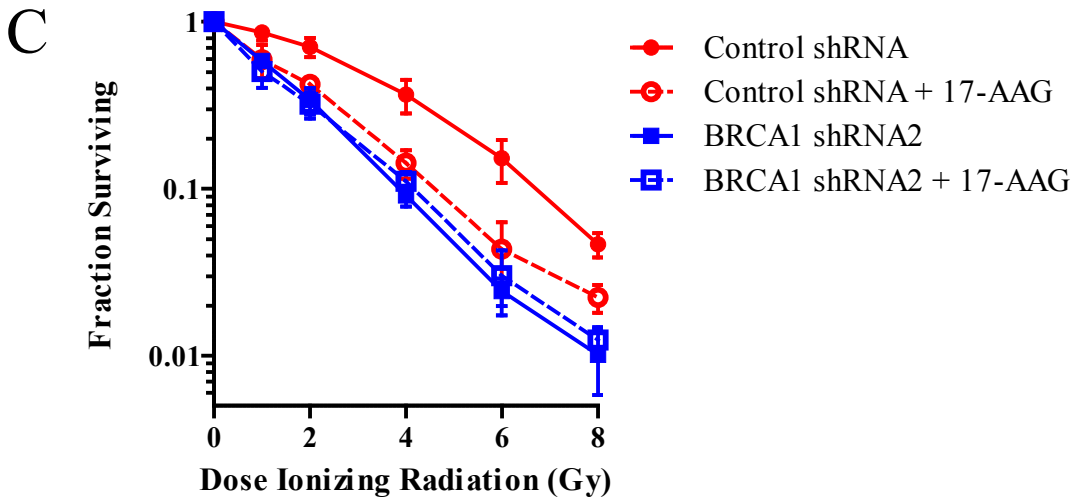

nM 17-AAG
400
300
200
100
0
***
HCC1937
HCC1037BRCA1

Figure 4. Wild-type *BRCA1* complementation confers resistance to 17-AAG in HCC1937 cells. (A) MTS assay of HCC1937 and $HCC1937^{BRCA1}$ cells treated with increasing concentrations of 17-AAG. (B) IC_{50} values based on data presented in panel A. Error bars represent SEM of three independent experiments. *p<0.05, **p<0.01, ***p<0.001 (student's t-test).


A
Dose IR (Gy)
0
1
2
4
6
Control + DMSO
Control + 17-AAG
shRNA2 + DMSO
shRNA2 + 17-AAG
B
0 Gy IR
n.s.
n.s.
Relative Clonogenicity
150
100
50
0
Control + DMSO
Control + 17-AAG
shRNA2 + DMSO
shRNA2 + 17-AAG


C
Fraction Surviving
1
0.1
0.01
0
2
4
6
8
Dose Ionizing Radiation (Gy)
Control shRNA
Control shRNA + 17-AAG
BRCA1 shRNA2
BRCA1 shRNA2 + 17-AAG

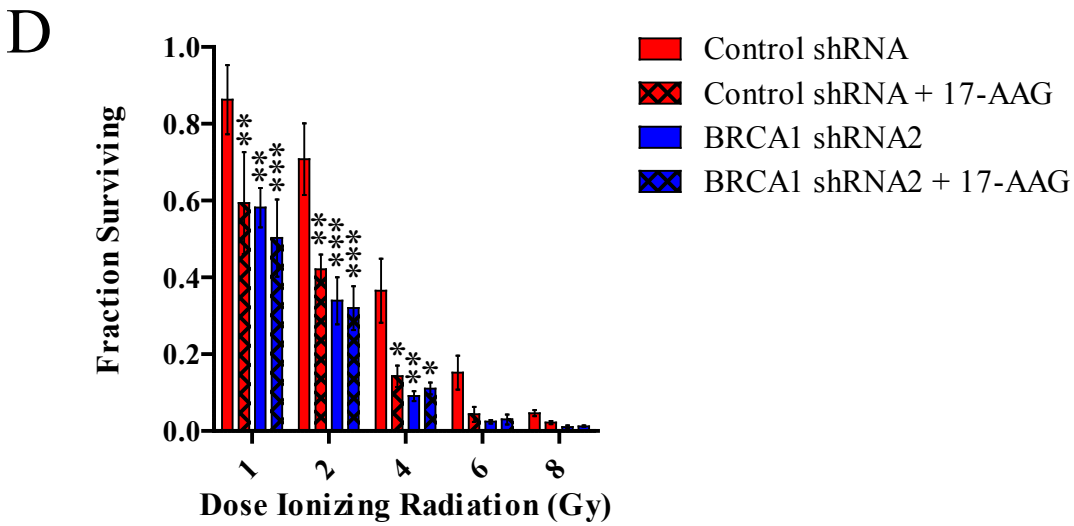

D
Fraction Surviving
1.0
0.8
0.6
0.4
0.2
0.0
1
2
4
6
8
Dose Ionizing Radiation (Gy)
Control shRNA
Control shRNA + 17-AAG
BRCA1 shRNA2
BRCA1 shRNA2 + 17-AAG

Figure 5. Loss of BRCA1 is epistatic to the ability of 17-AAG to potentiate MCF7 cells to ionizing radiation. (A) Representative images of control- or BRCA1-shRNA lentivirally ablated MCF7 cells in clonogenic growth assays 10 days after exposure to 0-6 Gy of IR in the presence or absence of 17-AAG. (B) Relative clonogenic potential of each population in the absence of IR. Error bars represent SEM of three independent experiments. n.s. = not significant. (C) Clonogenicity assay of control shRNA and BRCA1 shRNA2 infected MCF7 cells pre-treated with vehicle or 10 nM 17-AAG for two days and exposed to 0-6 Gy IR. Percent surviving at each dose of IR is relative to colonies formed at 0 Gy for each particular clone. (D) Analysis of survival based on data presented in panel C. Error bars represent SEM of three independent experiments. *$p<0.05$, **$p<0.01$, ***$p<0.001$ comparing each treatment to the control shRNA infected cells treated with vehicle (ANOVA, followed by Bonferroni post-test).

BRCA1-mutant and wild-type complemented breast and ovarian cancer cells (HCC1937 and UWB1.289, respectively). As expected, complementation of wild-type *BRCA1* (HCC1937^{BRCA1} and UWB1.289^{BRCA1}) increased resistance to carboplatin in both models (Figure 6A-D). HCC1937, HCC1937^{BRCA1}, UWB1.289 and UWB1.289^{BRCA1} cells were treated with combinations of 17-AAG and carboplatin and assessed by MTS assay to evaluate synergy. Our studies of combinatorial drug effect were performed in CalcuSyn and are based on the reasoning detailed in Appendix A.

17-AAG increased sensitivity to carboplatin in all cell lines (Figure 7A-C). Viability data at all equal-ratio combinations (Figure 7A-B, dotted boxes) enabled mathematical quantification of synergy as a combination index (CI) based on Equation 15 (Appendix A). 17-AAG and carboplatin were highly synergistic in HCC1937 and HCC1937^{BRCA1} cells, though the interaction of the two drugs in both mutant and wild-type complemented UWB1.289 cells appeared to be only additive (Figures 8 and 9). In the HCC1937 model, synergy was generally more pronounced in the wild-type complemented cell lines at the 100:1 and 1000:1 combination ratios (Figures 8 and 9). We interpret our findings to mean that the additional synergy observed in the wild-type complemented cell HCC1937 line is due to abrogation of BRCA1-dependent repair of carboplatin interstrand crosslinks. The lack of synergistic activity in the UWB1.289 cell model may be explained by a report indicating that complementation of wild-type *BRCA1* in this cell line only partially corrects the DNA damage response (DelloRusso et al., 2007). This is consistent with our data showing a modest and non-significant increase in resistance to carboplatin in the *BRCA1* wild-type complemented UWB1.289 cell line (Figure 6C-D).

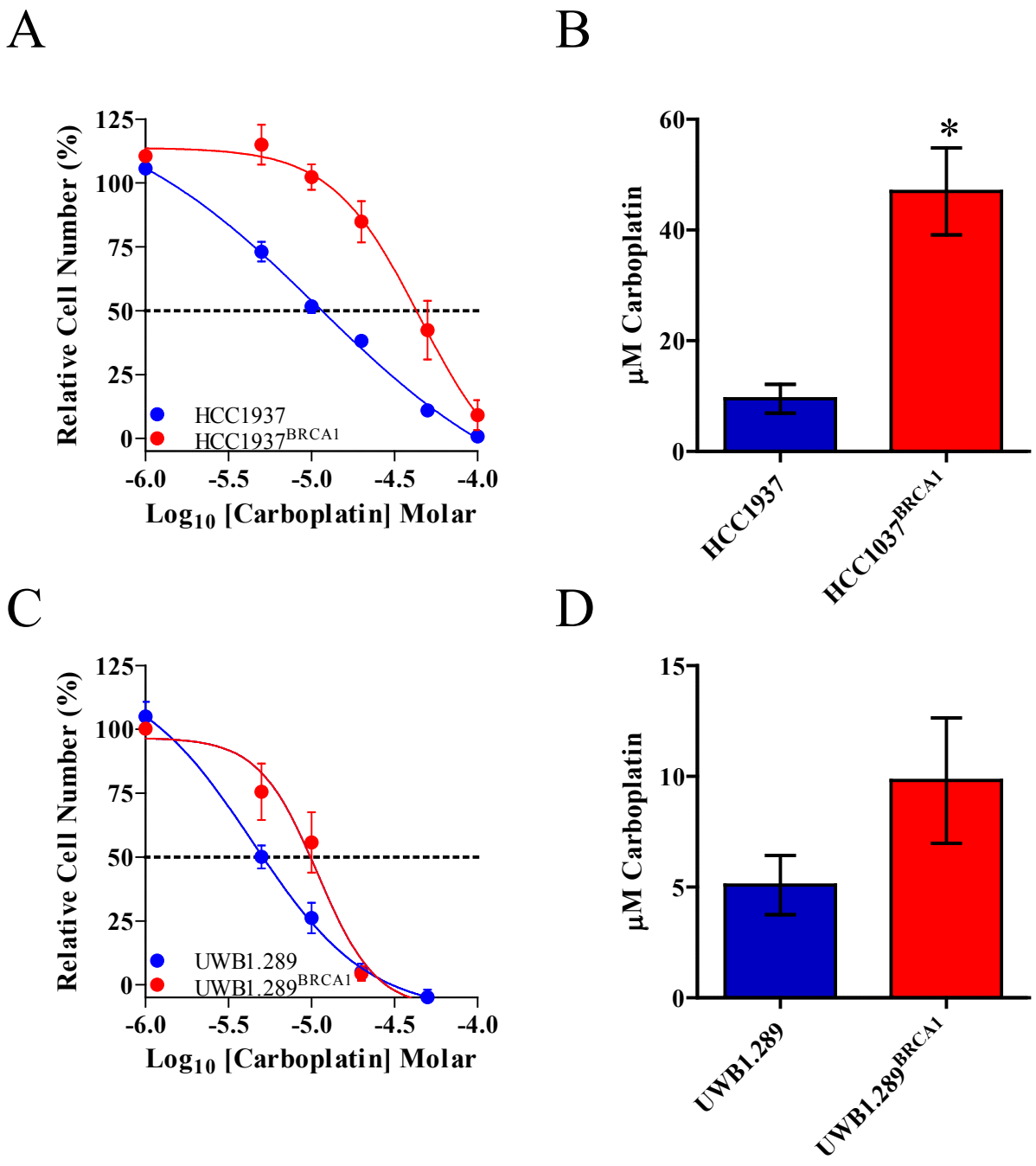

A
B
C
D
Relative Cell Number (%)
125
100
75
50
25
0
HCC1937
HCC1937BRCA1
-6.0
-5.5
-5.0
-4.5
-4.0
Log10 [Carboplatin] Molar
µM Carboplatin
60
40
20
0
*
HCC1937
HCC1037BRCA1
UWB1.289
UWB1.289BRCA1
15
10
5

Figure 6. Wild-type *BRCA1* complementation confers resistance to carboplatin in HCC1937 and UWB1.289 cells. MTS assay of HCC1937 and HCC1937^{BRCA1} cells (A) and UWB1.289 and UWB1.289^{BRCA1} cells (C) treated with increasing concentrations of carboplatin. (B and D) IC_{50} values based on data presented in panel A (B) and C (D). Error bars represent SEM of three independent experiments. *p<0.05, **p<0.01, ***p<0.001 (student's t-test).

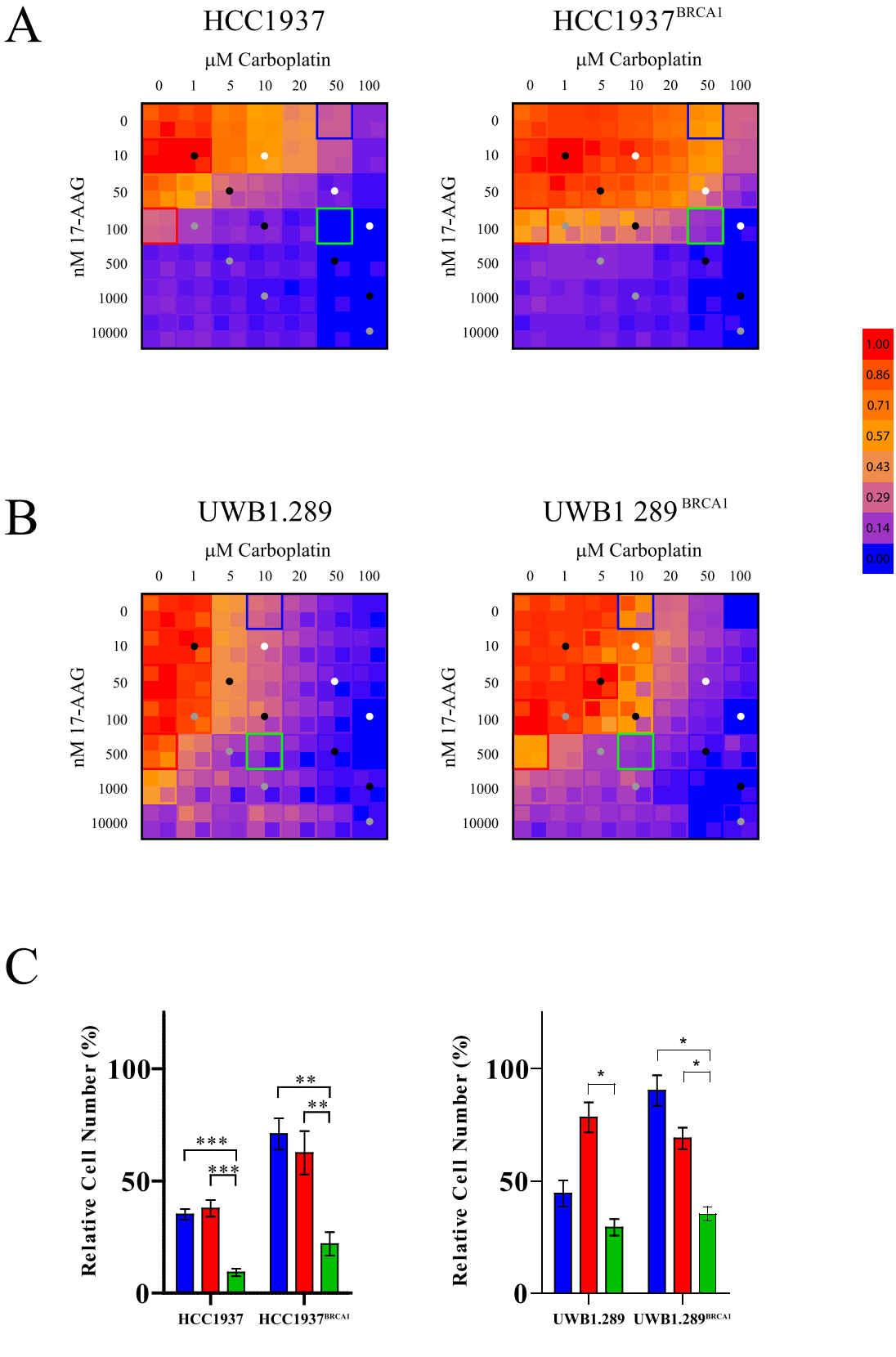
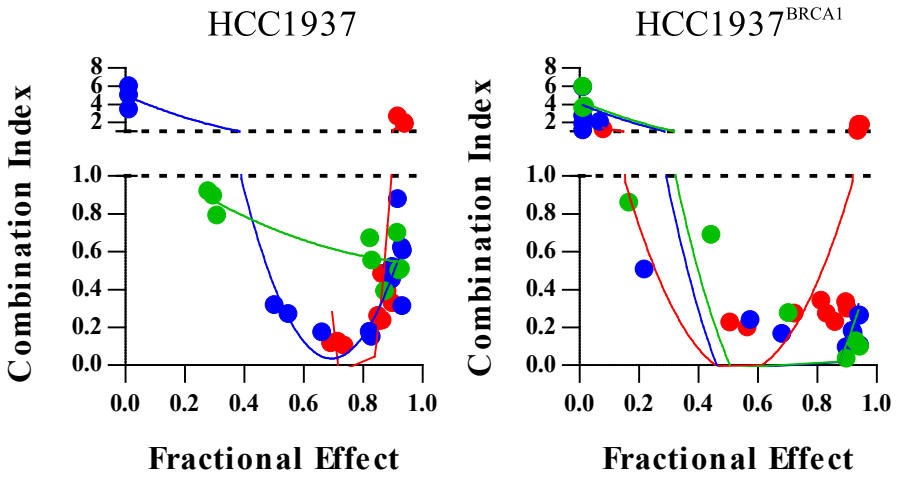
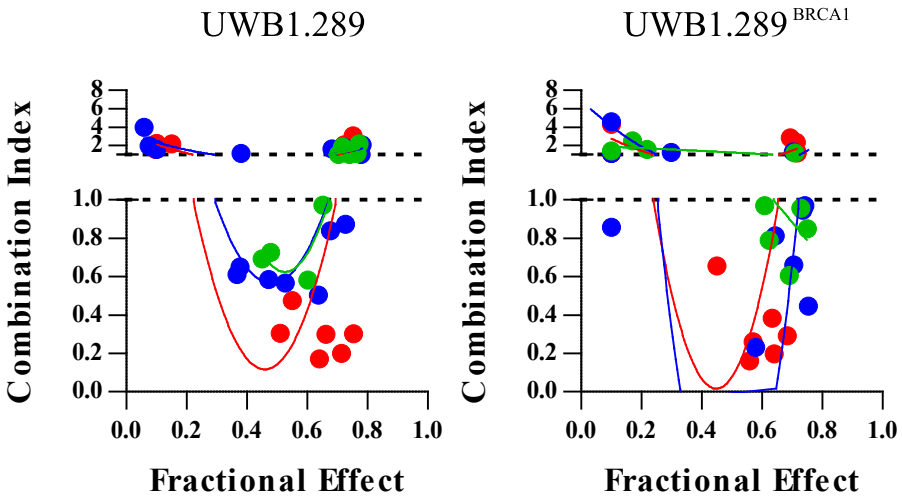

A
HCC1937
HCC1937BRCA1
µM Carboplatin
0 1 5 10 20 50 100
nM 17-AAG
0 10 50 100 500 1000 10000
1.00 0.86 0.71 0.57 0.43 0.29 0.14 0.00
B
UWB1.289
UWB1 289 BRCA1
C
Relative Cell Number (%)
100
50
0
***
***
**
**
HCC1937
HCC1937BRCA1
*
*
*
UWB1.289
UWB1.289BRCA1

Figure 7. Combination studies of 17-AAG and carboplatin in *BRCA1*-mutant and wild-type-complemented breast and ovarian cancer cells. (A and B) Heat maps of HCC1937 and HCC1937^{BRCA1} (A) and UWB1.289 and UWB1.289^{BRCA1} proliferation in response to various combinations of 17-AAG and carboplatin. Inlayed boxes denote mean plus standard deviation (upper left) or mean minus standard deviation (lower right). Colored dots denote equal ratio of carboplatin:17-AAG (10:1 (gray), 100:1 (black), 1000:1 (white)). (C) Proliferation of HCC1937 and HCC1937^{BRCA1} cells in response to 50 μM carboplatin, 100 nM 17-AAG or both, and UWB1.289 and UWB1.289^{BRCA1} cells in response to 10 μM carboplatin, 500 nM 17-AAG or both (denoted by blue-, red- and green-outlined boxes in panels A and B). Error bars represent SEM of three independent experiments. *$p<0.05$, **$p<0.01$, ***$p<0.001$ (student's t-test).

A


HCC1937
HCC1937BRCA1
Combination Index
Fractional Effect
8
6
4
2
1.0
0.8
0.6
0.4
0.2
0.0
0.0
0.2
0.4
0.6
0.8
1.0

B


UWB1.289
UWB1.289 BRCA1
Combination Index
Fractional Effect
8
6
4
2
1.0
0.8
0.6
0.4
0.2
0.0
0.0
0.2
0.4
0.6
0.8
1.0

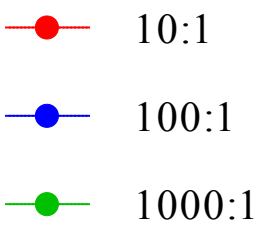

10:1
100:1
1000:1

Figure 8. Plots of combination index (CI) versus fractional effect in HCC1937 and HCC1937^{BRCA1} cells (A) and UWB1.289 and UWB1.289^{BRCA1} cells; solid lines denote second order polynomial non-linear regression. Dotted line (y=1) represents additivity, whereas y<1 indicates synergy and y>1 indicates antagonism.

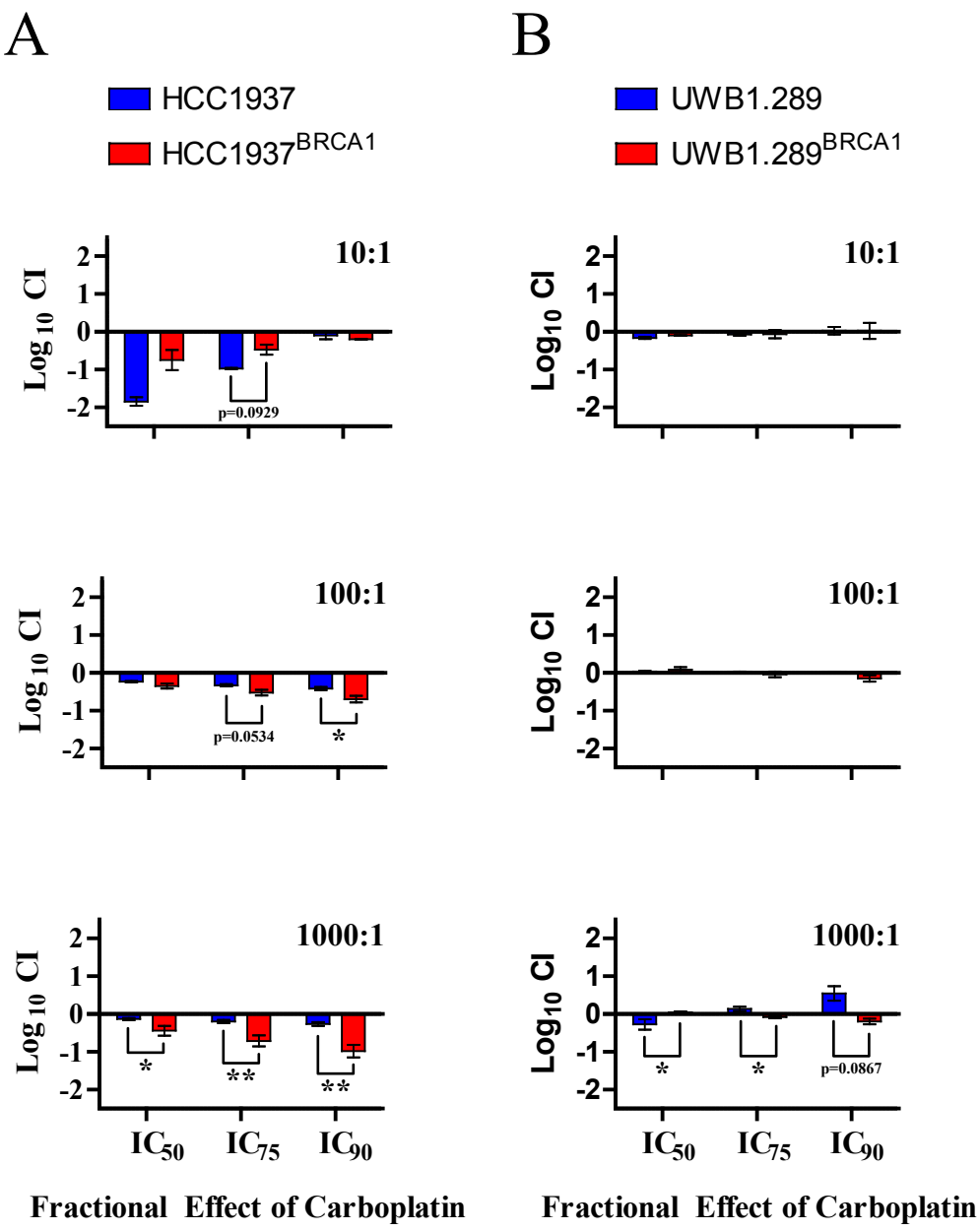

A
B
HCC1937
HCC1937BRCA1
UWB1.289
UWB1.289BRCA1
10:1
100:1
1000:1
Log10 CI
p=0.0929
p=0.0534
*
**
p=0.0867
IC50
IC75
IC90
Fractional Effect of Carboplatin
Fractional Effect of Carboplatin

Figure 9. Combination indices of 17-AAG and carboplatin at the IC_{50}, IC_{75} and IC_{90} fractional effect levels of carboplatin for the 10:1, 100:1 and 1000:1 carboplatin:17-AAG ratios in HCC1937 and $HCC1937^{BRCA1}$ cells (A) and UWB1.289 and $UWB1.289^{BRCA1}$ cells (B). Data were logarithmically transformed for ease of interpretation, where $\log_{10}$ CI=0 indicates additivity, $\log_{10}$ CI<0 indicates synergy and $\log_{10}$ CI>0 indicates antagonism. Error bars represent SEM of three independent experiments. *p<0.05, **p<0.01, ***p<0.001 based on linear CI values comparing the *BRCA1*-wild-type complemented sample to the corresponding *BRCA1*-mutant sample for each effect level and ratio (student's t-test).

To evaluate the ability of 17-AAG to resensitize non-*BRCA1*-mutant ovarian cancer cells, we employed the A2780 cell line and two platinum-resistant clones of this line designated CP70 and C30. The carboplatin IC_{50} in A2780 cells was noted to be 46 μM, while the CP70 and C30 cell lines had not reached IC_{50} by 100 μM (Figure 10A). Sensitivity to 17-AAG was not significantly different between A2780 cells and either resistant clone, though the C30 clone did exhibit significant 17-AAG resistance compared to the CP70 clone (Figure 10B). Consistent with our previous studies, treatment with 17-AAG abolished expression of BRCA1 and other described HSP90 client proteins involved in homologous recombination, including BRCA2, CHK1 and RAD51 (Figure 10C). As before, combinatorial treatment of A2780, C70 and C30 cells with carboplatin and 17-AAG was performed to evaluate whether 17-AAG could resensitize platinum-resistant sporadic ovarian cancer cells. In A2780 and C30 cells, combinatorial treatment with 17-AAG and carboplatin resulted in greater suppression of cell growth than did either agent alone, while in CP70 cells, the bulk of the therapeutic effect appeared to be due to the action of 17-AAG alone (Figure 11A-B). Quantification of the *CI* did reveal synergy in all three cell lines at nearly all drug ratios and effect levels, though only the C30 clone exhibited enhanced synergy at the 100:1 and 1000:1 ratios when compared to the parental A2780 cell line (Figures 12 and 13).

Wild-Type BRCA1 Prevents Mitotic Entry After HSP90 Inhibition

In addition to coordinating the repair of DSBs, BRCA1 also regulates multiple aspects of cell cycle progression and induces the G2/M checkpoint to prevent perpetuation of genetic damage through mitosis. We sought to examine whether *BRCA1* status influenced activation of the

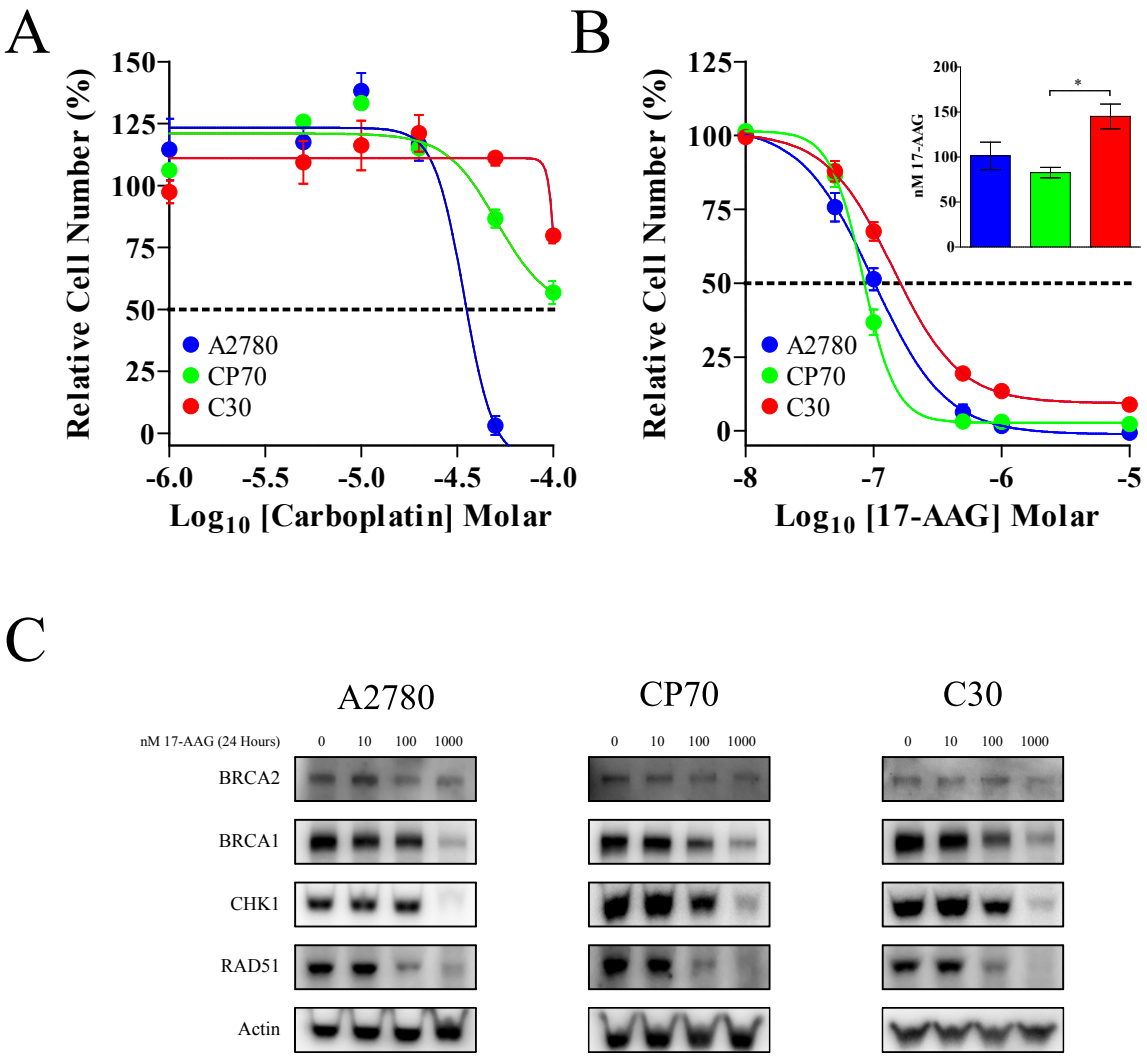
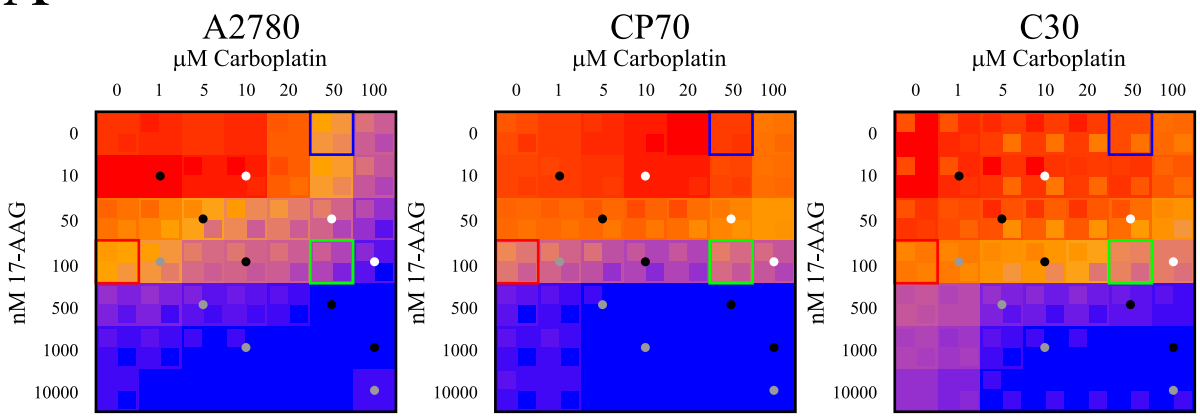

A
Relative Cell Number (%)
150
125
100
75
50
25
0
A2780
CP70
C30
-6.0
-5.5
-5.0
-4.5
-4.0
Log10 [Carboplatin] Molar
B
Relative Cell Number (%)
125
100
75
50
25
0
A2780
CP70
C30
-8
-7
-6
-5
Log10 [17-AAG] Molar
nM 17-AAG
200
150
100
50
0
*
C
A2780
CP70
C30
nM 17-AAG (24 Hours)
0
10
100
1000
BRCA2
BRCA1
CHK1
RAD51
Actin

Figure 10. Sensitivity of sporadic platinum-sensitive (A2780) and platinum-resistant (CP70 and C30) ovarian cancer cells to carboplatin and 17-AAG. MTS assay of A2780, CP70 and C30 cells treated with increasing concentrations of carboplatin (A) or 17-AAG (B). Inlayed graph in panel B denotes IC_{50} values. Error bars represent SEM of three independent experiments. *$p<0.05$, **$p<0.01$, ***$p<0.001$ (student's t-test). (C) Cells were treated with the indicated concentrations of 17-AAG for 24 hours and western blots were performed for BRCA1 and other HR-associated proteins.

A


A2780
µM Carboplatin
0 1 5 10 20 50 100
nM 17-AAG
0
10
50
100
500
1000
10000
CP70
µM Carboplatin
0 1 5 10 20 50 100
nM 17-AAG
0
10
50
100
500
1000
10000
C30
µM Carboplatin
0 1 5 10 20 50 100
nM 17-AAG
0
10
50
100
500
1000
10000

B

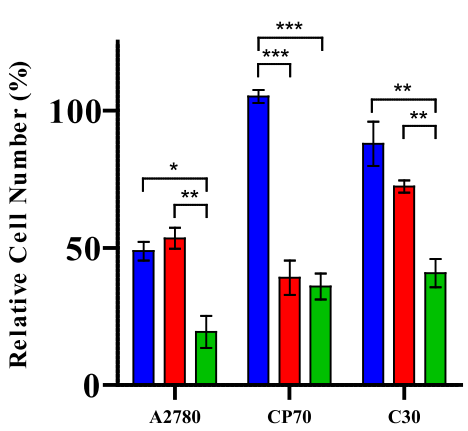

Relative Cell Number (%)
100
50
0
*
**
***
***
**
**
A2780
CP70
C30

Figure 11. Combination studies of 17-AAG and carboplatin in sporadic platinum-sensitive and -resistant ovarian cancer cells. (A) Heat maps of A2780, CP70 and C30 proliferation in response to various combinations of 17-AAG and carboplatin. Inlayed boxes denote mean plus standard deviation (upper left) or mean minus standard deviation (lower right). Colored dots denote equal ratio of carboplatin:17-AAG (10:1 (gray), 100:1 (black), 1000:1 (white)). (B) Proliferation of A2780, CP70 and C30 cells in response to 50 μM carboplatin, 100 nM 17-AAG or both (denoted by blue-, red- and green-outlined boxes in panel A). Error bars represent SEM of three independent experiments; *$p<0.05$, **$p<0.01$, ***$p<0.001$ (student's t-test).

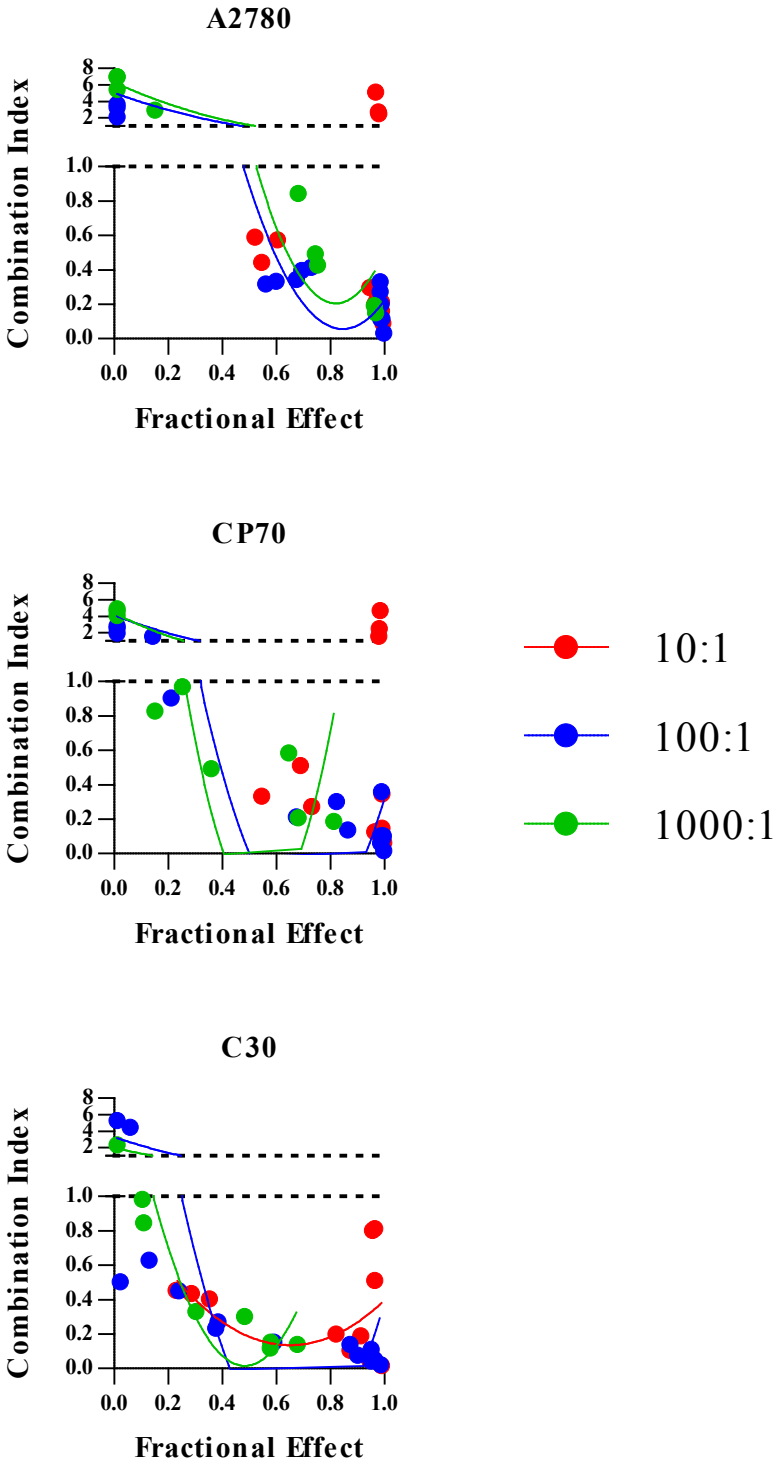
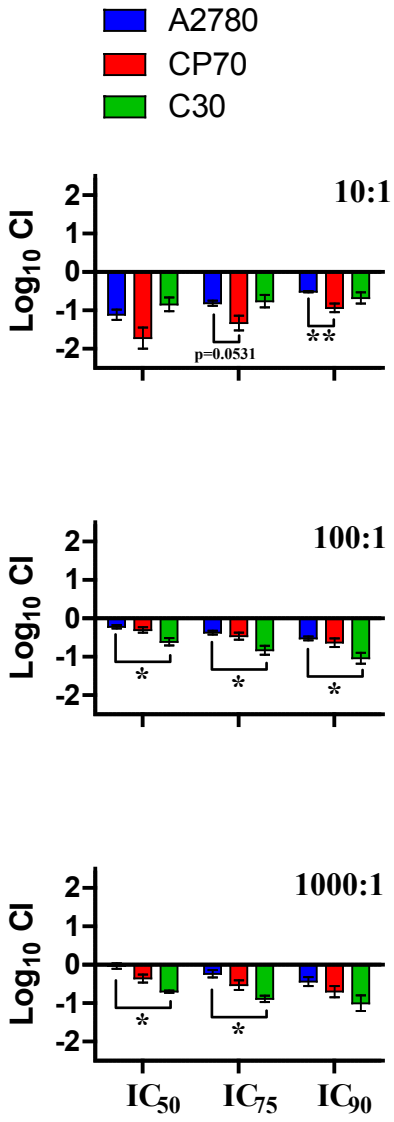

A2780
Combination Index
Fractional Effect
CP70
Combination Index
Fractional Effect
C30
Combination Index
Fractional Effect
10:1
100:1
1000:1

Figure 12. Plots of combination index (CI) versus fractional effect in A2780, CP70 and C30 cells; solid lines denote second order polynomial non-linear regression. Dotted line (y=1) represented additivity, whereas y<1 indicates synergy and y>1 indicates antagonism.



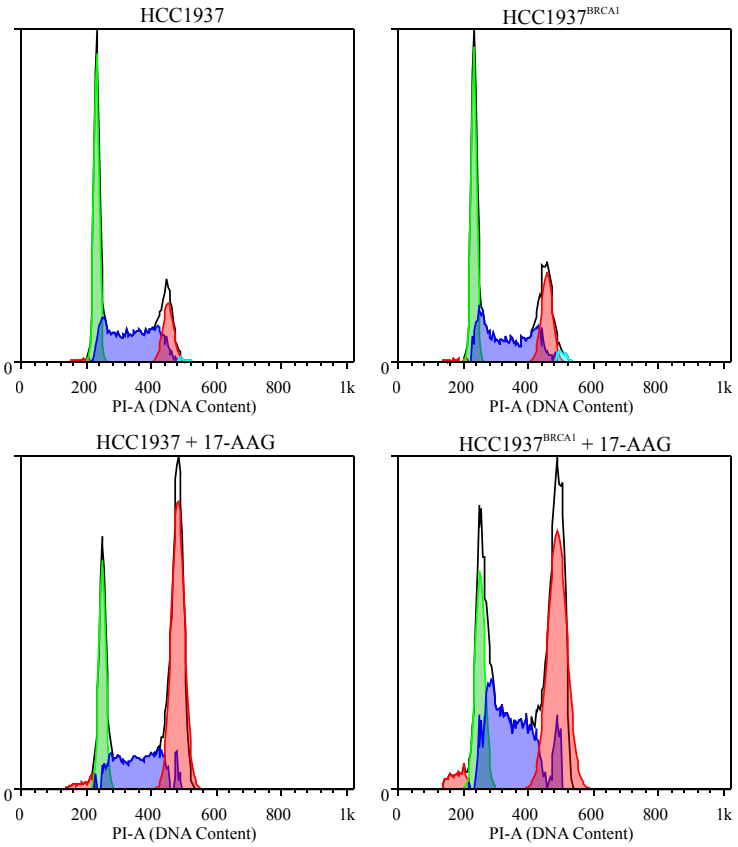
Fractional Effect of Carboplatin

Figure 13. Combination indices of 17-AAG and carboplatin at the IC_{50}, IC_{75} and IC_{90} fractional effect levels of carboplatin for the 10:1, 100:1 and 1000:1 carboplatin:17-AAG ratios in A2780, CP70 and C30 cells. Data were logarithmically transformed for ease of interpretation, where $\log_{10}$ CI=0 indicates additivity, $\log_{10}$ CI<0 indicates synergy and $\log_{10}$ CI>0 indicates antagonism. Error bars represent SEM of three independent experiments. *p<0.05, **p<0.01, ***p<0.001 based on linear CI values comparing the platinum-resistant (CP70 or C30) samples to the platinum-sensitive parental A2780 sample for each effect level and ratio (student's t-test).

G2/M checkpoint and whether changes in DNA synthesis and/or drug-induced apoptosis were responsible for differential sensitivity of *BRCA1* wild-type and mutant cell lines to 17-AAG.

In both HCC1937 and HCC1937^{BRCA1} cells, treatment with 17-AAG induced a pronounced G2/M arrest at 24 hours (Figure 14A-B). To discriminate whether cells were arresting in G2 or M phase, we performed cell cycle analysis with costaining for pH3^{S28}, a histone modification only present during mitosis. Upon treatment with 17-AAG, pH3^{S28} staining increased from 3.9% to 38.0% in the *BRCA1* mutant HCC1937 cells, compared to a much more modest increase from 3.0% to 7.9% in the wild-type complemented HCC1937^{BRCA1} cells (Figure 15A-B). We further evaluated the effects of 17-AAG on mitotic progression in HCC1937 and HCC1937^{BRCA1} cells in the presence and absence of radiation-induced DNA damage. Consistent with our flow cytometric findings, phosphorylation of H3^{S10}, another mitosis-associated histone phosphorylation event, and expression of the M-phase specific cyclin B1 were only induced in *BRCA1*-mutant HCC1937 cells after 17-AAG treatment (Figure 16). These data imply that wild-type BRCA1 is able to prevent 17-AAG-induced entry into M phase, while cells harbouring mutant *BRCA1* fail to activate the G2/M checkpoint and progress into mitosis despite the presence of DNA damage. BRCA1 activates the G2/M checkpoint by facilitating the phosphorylation of CHK1^{S345}, and in effect, by regulating the expression, phosphorylation and localization of CDC25C and the CDC2/cyclin B-complex (Yamane et al., 2007; Yarden et al., 2002). Because CHK1 is a known client protein of HSP90 (Arlander et al., 2003), we sought to understand whether 17-AAG induced G2/M arrest depended on the ability of BRCA1 to activate CHK1. Consistent with previous reports, 17-AAG treatment of both cell lines destabilized CHK1 (Figure 16). As expected, radiation-induced phosphorylation of CHK1^{S345} was much

A


HCC1937
HCC1937BRCA1
HCC1937 + 17-AAG
HCC1937BRCA1 + 17-AAG
0
200
400
600
800
1k
PI-A (DNA Content)

B

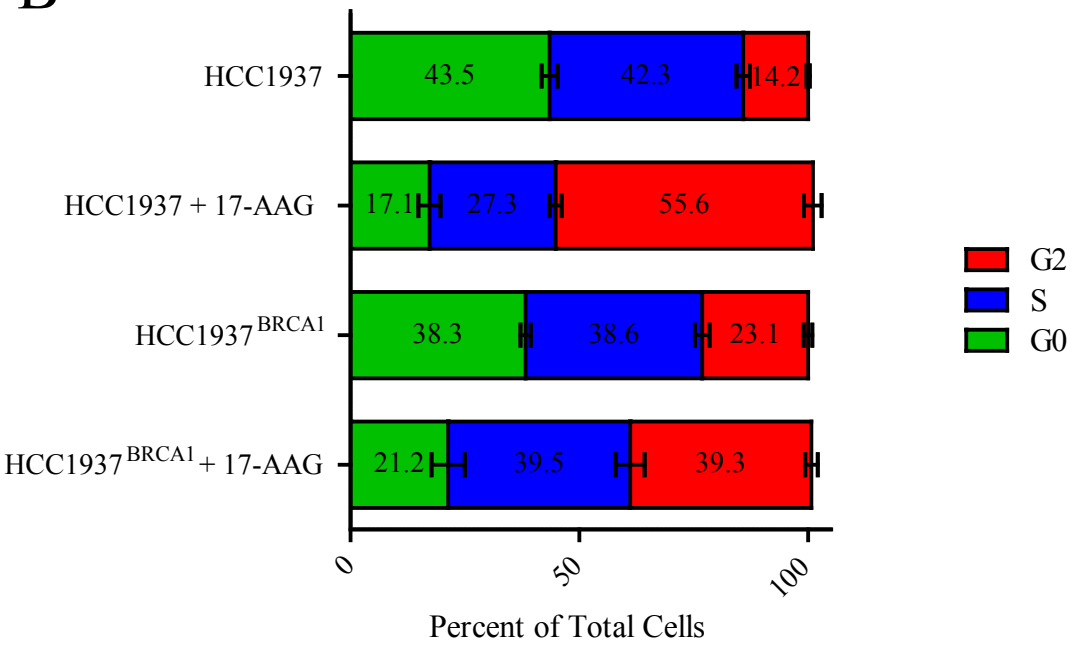
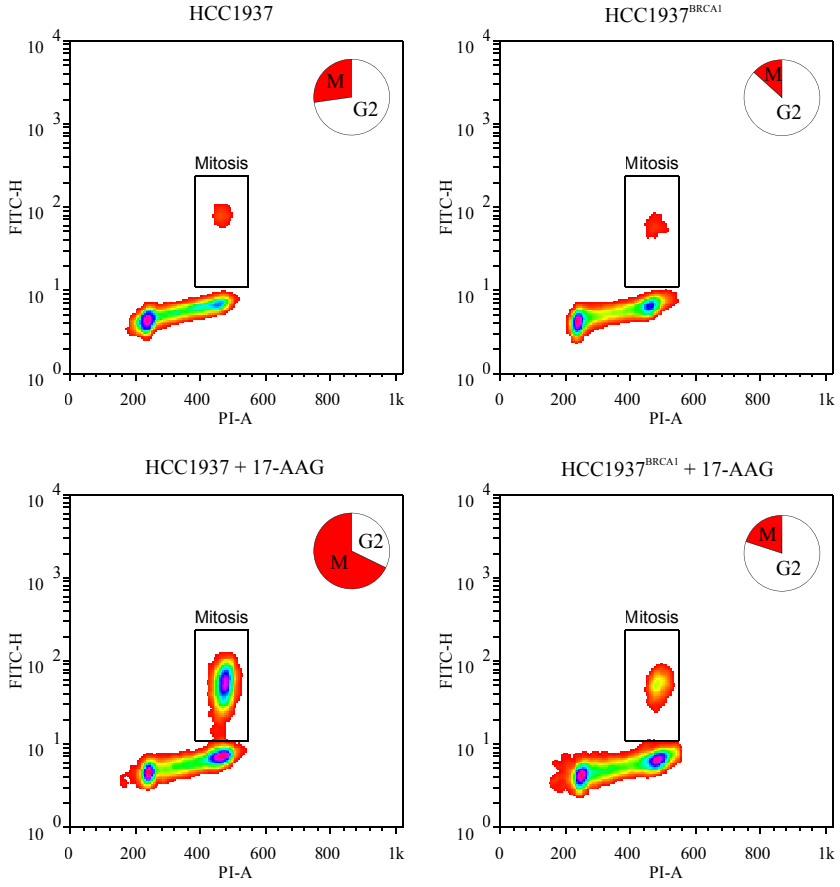

HCC1937
HCC1937 + 17-AAG
HCC1937BRCA1
HCC1937BRCA1 + 17-AAG
43.5
42.3
14.2
17.1
27.3
55.6
38.3
38.6
23.1
21.2
39.5
39.3
0
50
100
Percent of Total Cells
G2
S
G0

Figure 14. (A) Flow cytometric evaluation of cell cycle distribution in HCC1937 and HCC1937^{BRCA1} cells after 24 hours of exposure to 250 nM 17-AAG. (B) Graphical representation of distribution; error bars represent SEM in three independent experiments.

A


HCC1937
HCC1937BRCA1
HCC1937 + 17-AAG
HCC1937BRCA1 + 17-AAG
M
G2
Mitosis
FITC-H
PI-A

B

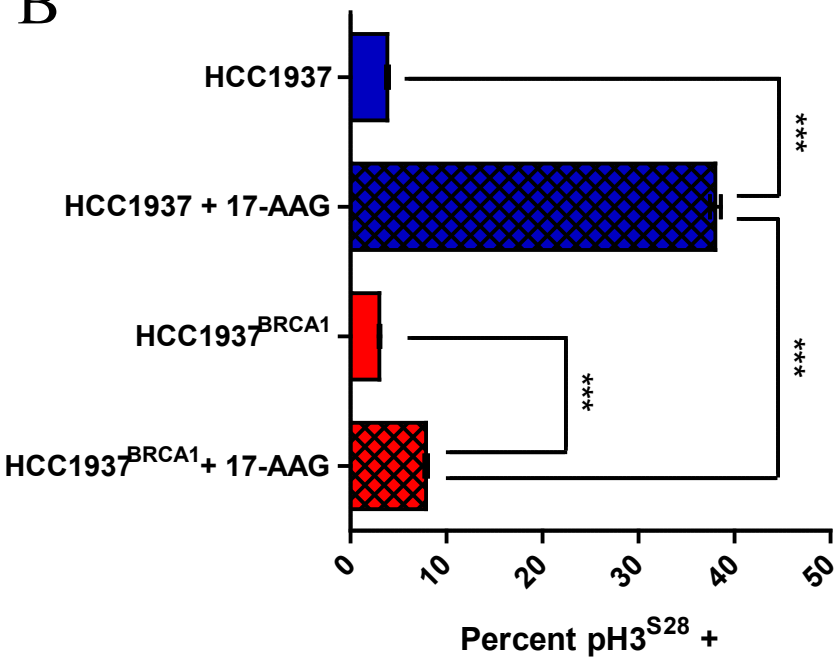

HCC1937
HCC1937 + 17-AAG
HCC1937BRCA1
HCC1937BRCA1+ 17-AAG
***
***
***
0
10
20
30
40
50
Percent pH3S28 +

Figure 15. (A) Flow cytometric evaluation of $pH3^{S28}$ staining in HCC1937 and $HCC1937^{BRCA1}$ cells after 24 hours of exposure to 250 nM 17-AAG. (B) Graphical representation of $pH3^{S28}$-positivity; error bars represent SEM in three independent experiments. *$p<0.05$, **$p<0.01$, ***$p<0.001$ (student's t-test).

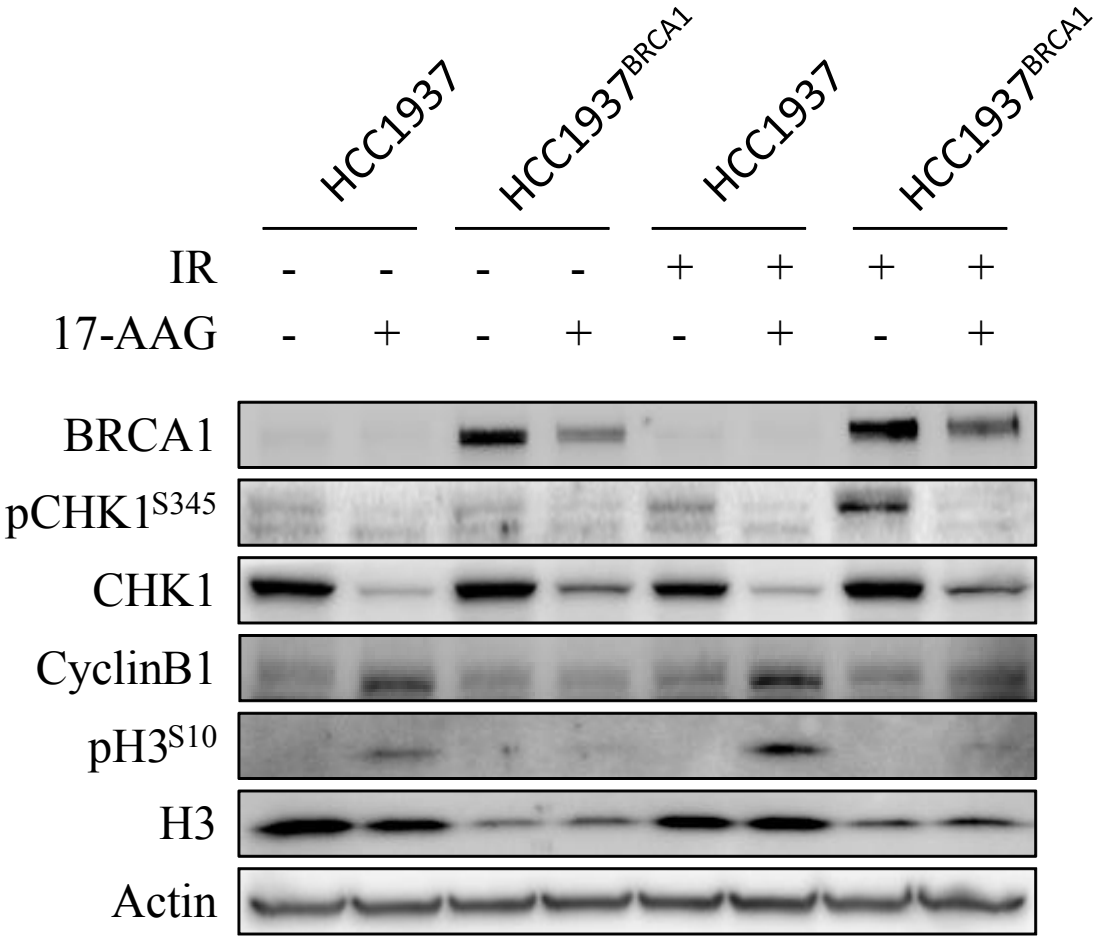

HCC1937
HCC1937BRCA1
HCC1937
HCC1937BRCA1
IR - - - - + + + +
17-AAG - + - + - + - +
BRCA1
pCHK1S345
CHK1
CyclinB1
pH3S10
H3
Actin

Figure 16. BRCA1 prevents 17-AAG-induced mitotic entry independently of CHK1. Western blots for cycle checkpoint and mitosis-associated proteins in HCC1937 and HCC1937^{BRCA1} cells after 24 hours of exposure to 250 nM 17-AAG, 10 Gy ionizing radiation or both.

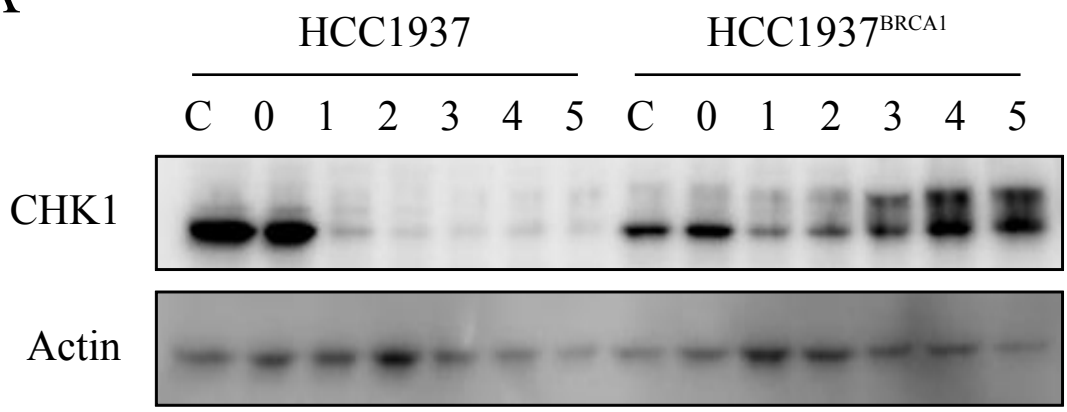
more robust in HCC1937^{BRCA1} cells than in HCC1937 cells, and 17-AAG interfered with this phorphorylation event (Figure 16). HCC1937^{BRCA1} cells did not induce H3^{S10} phorphorylation or cyclin B1 expression in response to 17-AAG in the presence or absence of radiation, implying that BRCA1 prevents 17-AAG induced mitotic entry independently of its well-characterized role in promoting CHK1^{S345} phosphorylation (Figure 16). Interestingly, we did note that 17-AAG did not destabilize CHK1 to the same extent in HCC1937^{BRCA1} cells as it did in *BRCA1* mutant HCC1937 cells (Figures 16 and 17).

17-AAG Selectively Reduces Replication Capacity in BRCA1-Mutant Cells

Cell cycle analysis also indicated that 17-AAG selectively reduced DNA synthesis in the *BRCA1* mutant HCC1937 cells (Figure 14A-B). To confirm this, we performed EdU incorporation assays following 17-AAG treatment. Loss of BRCA1 has been shown to accelerate growth in tumor cells (Thompson et al., 1995). Consistent with these reports, HCC1937^{BRCA1} cells incorporated EdU more slowly than the mutant HCC1937 cells (Figures 18A-B). Treatment with 17-AAG reduced DNA synthesis by nearly 50% in the mutant HCC1937 cells, while having no discernible effect on the wild-type complemented HCC1937^{BRCA1} cells (Figures 18A-B). The spontaneous accumulation of DSBs in response to 17-AAG (Chapter II Figure 10) will incite replication stress during S phase. Our data suggest that *BRCA1* mutant cells will acutely lose replication capacity in response to 17-AAG, as they are unable to resolve stalled replication structures, while those cells with wild-type *BRCA1* may be resistant to 17-AAG-induced replication stress, at least initially.

Loss of BRCA1 Enhances 17-AAG-Induced Apoptosis

A


HCC1937
HCC1937BRCA1
C 0 1 2 3 4 5 C 0 1 2 3 4 5
CHK1
Actin

B

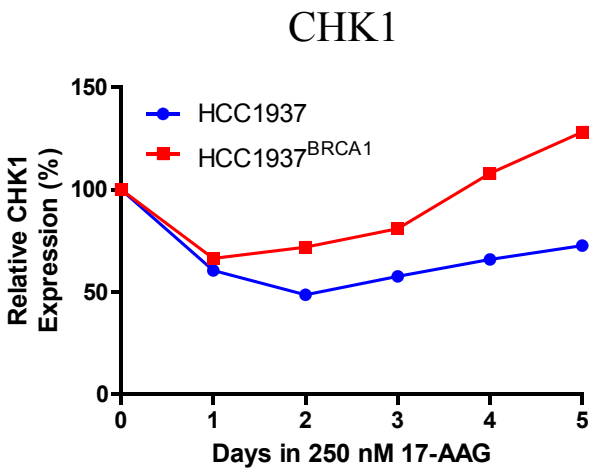
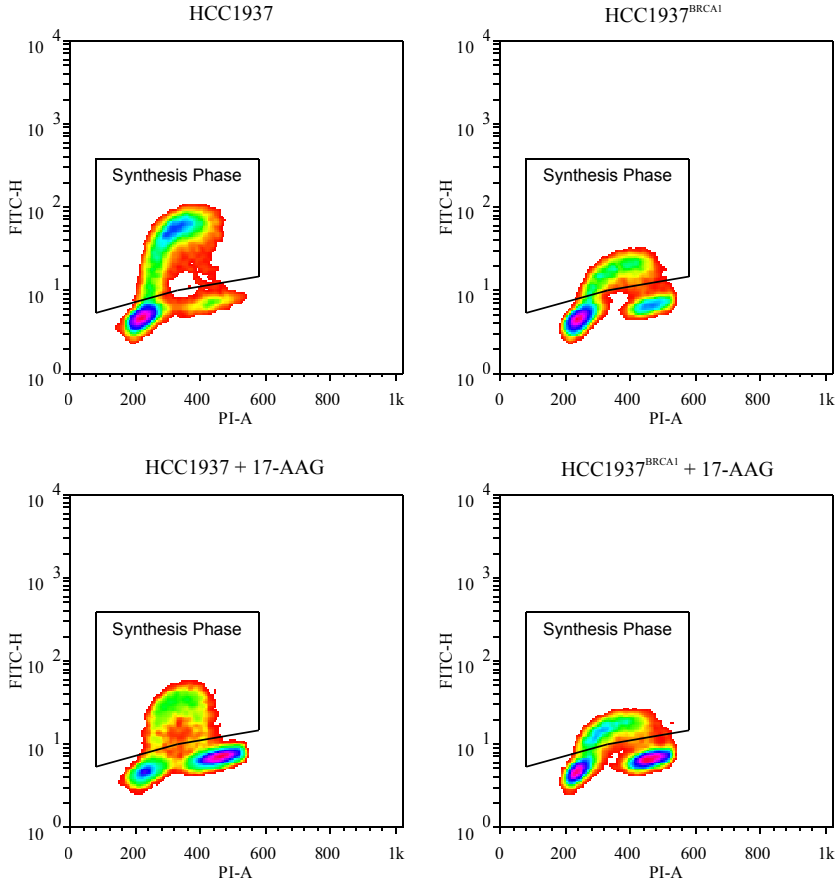

CHK1
HCC1937
HCC1937BRCA1
Relative CHK1 Expression (%)
150
100
50
0
0 1 2 3 4 5
Days in 250 nM 17-AAG

Figure 17. Wild-type BRCA1 expression mitigates 17-AAG-induced CHK1 degradation. (A) HCC1937 and HCC1937^{BRCA1} cells were grown in 250 nM 17-AAG for the indicated number of days (C indicates no treatment, '0' indicates vehicle control). Total protein was isolated, elecrophoresed and probed with the indicated antibodies. (B) Graphical representation of CHK1 protein levels in HCC1937 and HCC1937^{BRCA1} cells following 17-AAG treatment. Density of CHK1 band was normalized to corresponding density of actin band, and then all timepoints were normalized to the vehicle control lane.

A


HCC1937
HCC1937BRCA1
HCC1937 + 17-AAG
HCC1937BRCA1 + 17-AAG
Synthesis Phase
FITC-H
PI-A

B

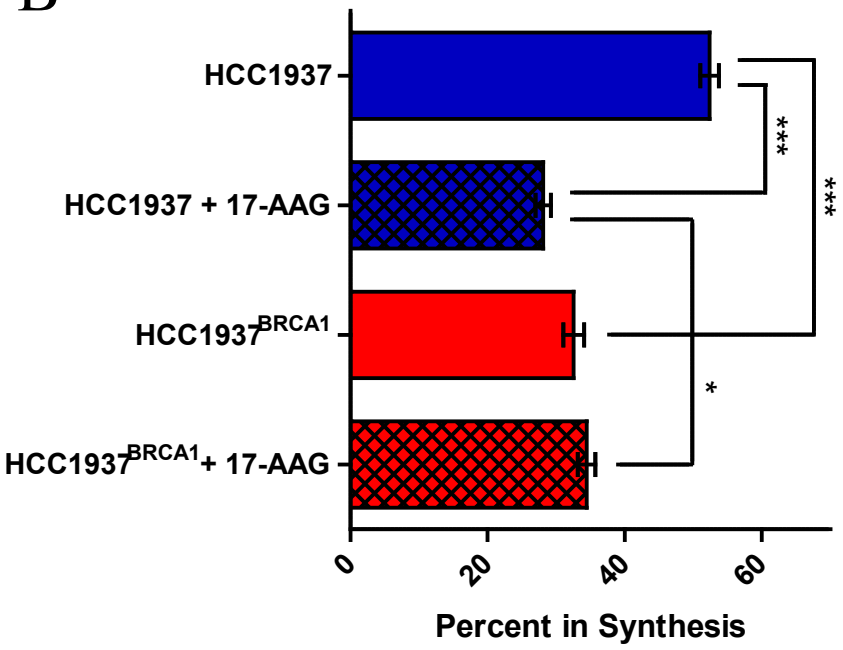
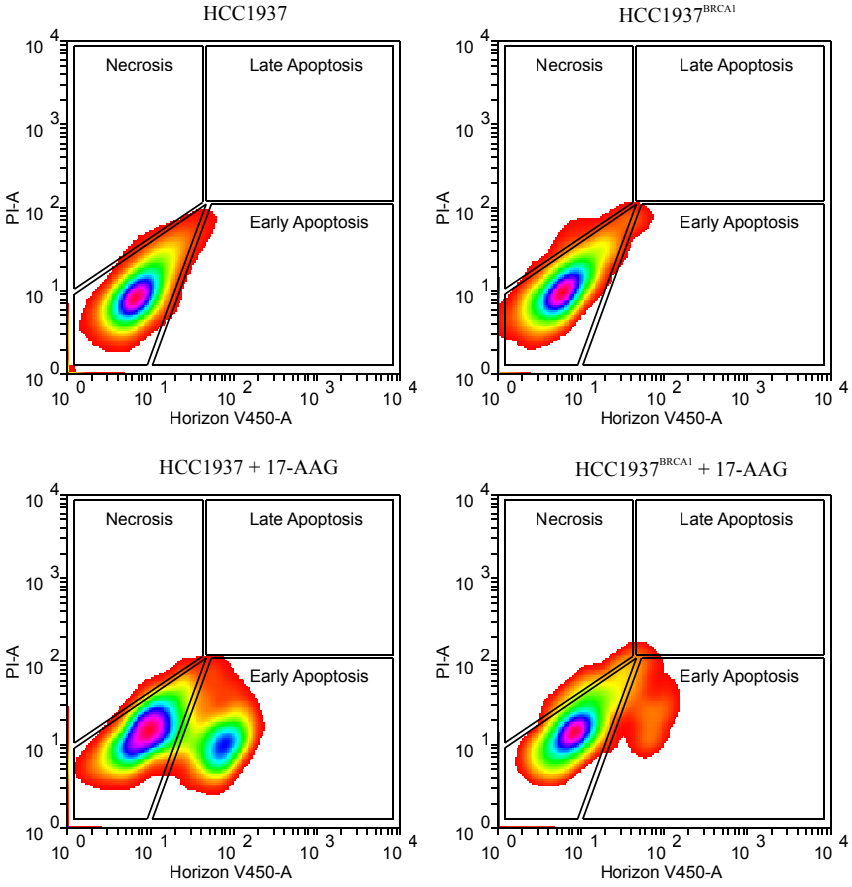

HCC1937
HCC1937 + 17-AAG
HCC1937BRCA1
HCC1937BRCA1+ 17-AAG
***
***
*
0
20
40
60
Percent in Synthesis

Figure 18. (A) Flow cytometric evaluation of EdU incorporation in HCC1937 and HCC1937^{BRCA1} cells after 24 hours of exposure to 250 nM 17-AAG. (B) Graphical representation of EdU incorporation; error bars represent SEM in three independent experiments. *$p<0.05$, **$p<0.01$, ***$p<0.001$ (student's t-test).

Though we observed dramatic BRCA1-dependent changes in mitotic progression and DNA synthesis in response to 17-AAG, we sought to understand the contribution of cell death to the hypersensitivity phenotype observed in *BRCA1*-mutant HCC1937 cells. Annexin V staining (a marker of early apoptosis) was performed at 24 hours after 17-AAG treatment and TUNEL staining (a marker of late apoptosis) at 24, 48 and 72 hours post treatment. Annexin V staining increased from 2.0% to 28.4% after 24 hours of 17-AAG treatment in HCC1937 cells, compared to an increase from 2.0% to 6.4% in HCC1937^{BRCA1} cells (Figures 19A-B). No difference in TUNEL staining was evident at 24 or 48 hours, but by 72 hours, the HCC1937 cells exhibited significantly more DNA fragmentation than the wild-type HCC1937^{BRCA1} cells (Figures 20A-B).

Treatment with N-terminal HSP90 inhibitors, like 17-AAG, disrupt the interaction between HSP90 and heat shock factor 1 (HSF1), and induce expression of stress responsive genes like HSP70 (Creagh et al., 2000; Morimoto, 1998; Zou et al., 1998). The inability to mount such a stress response has previously been associated with hypersensitivity to HSP90 inhibitors (Bagatell et al., 2000). To evaluate whether BRCA1 expression altered the heat shock response, HCC1937 and HCC1937^{BRCA1} cells were exposed to heat shock (42°C). In response to proteomic stress, HSF1 becomes phosphorylated by a number of kinases and induces expression of multiple genes involved in protection from thermal and other stresses (Holmberg et al., 2001). In both HCC1937 and HCC1937^{BRCA1} cells, the electrophoretic mobility of HSF1 shifted in a similar manner, and there were no observed differences in expression of the critical heat shock protein HSP70 (Figure 21). Conversely, it was noted that HSP27, a heat shock protein with significant anti-apoptotic activity was overexpressed in HCC1937^{BRCA1} cells, even in the absence

A


HCC1937
HCC1937BRCA1
HCC1937 + 17-AAG
HCC1937BRCA1 + 17-AAG
Necrosis
Late Apoptosis
Early Apoptosis
PI-A
Horizon V450-A

B

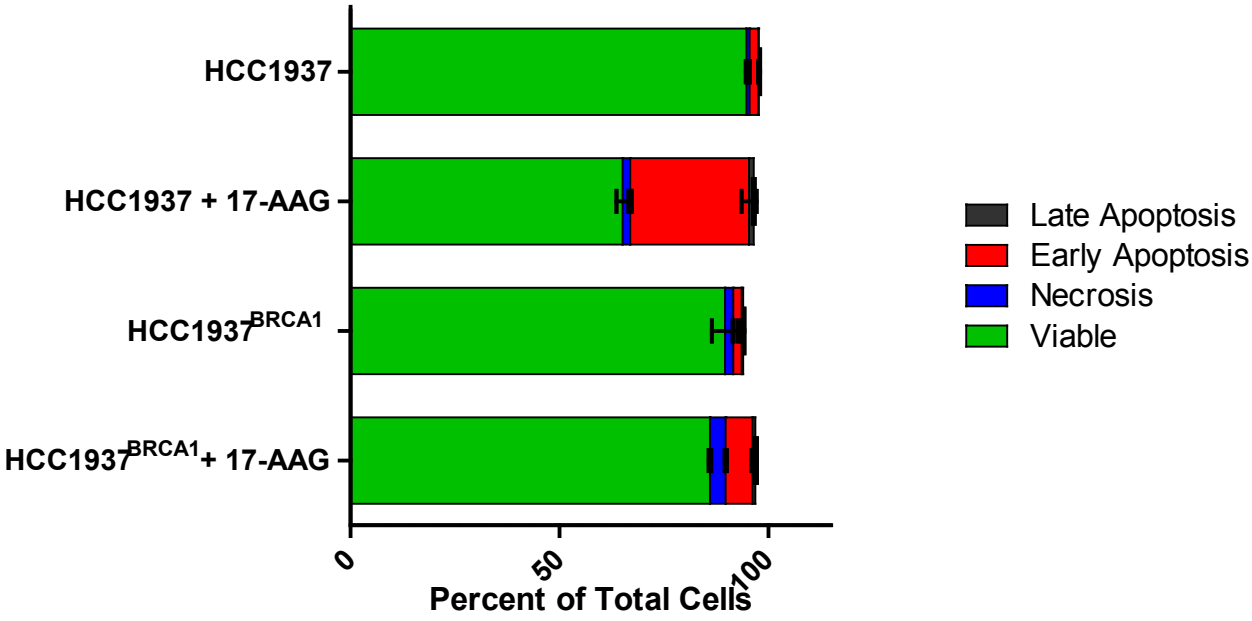
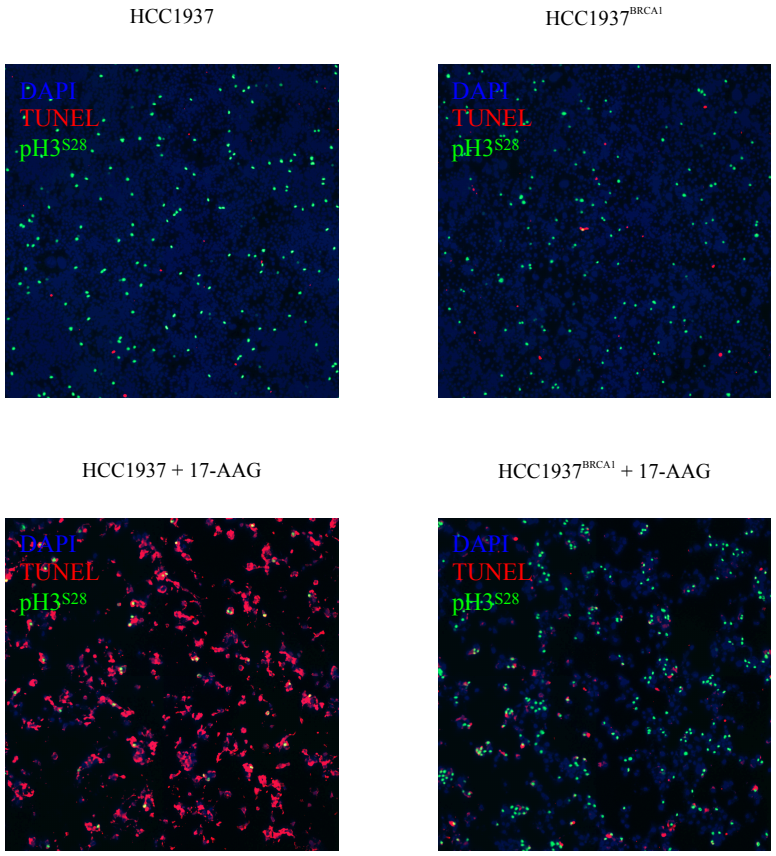

HCC1937
HCC1937 + 17-AAG
HCC1937BRCA1
HCC1937BRCA1+ 17-AAG
0
50
100
Percent of Total Cells
Late Apoptosis
Early Apoptosis
Necrosis
Viable

Figure 19. (A) Flow cytometric evaluation of apoptosis by Annexin V and propidium iodide staining in HCC1937 and HCC1937^{BRCA1} cells after 24 hours of exposure to 250 nM 17-AAG. (B) Graphical representation of apoptosis and necrosis; error bars represent SEM in three independent experiments.

A


HCC1937
HCC1937BRCA1
DAPI
TUNEL
pH3S28
HCC1937 + 17-AAG
HCC1937BRCA1 + 17-AAG

B

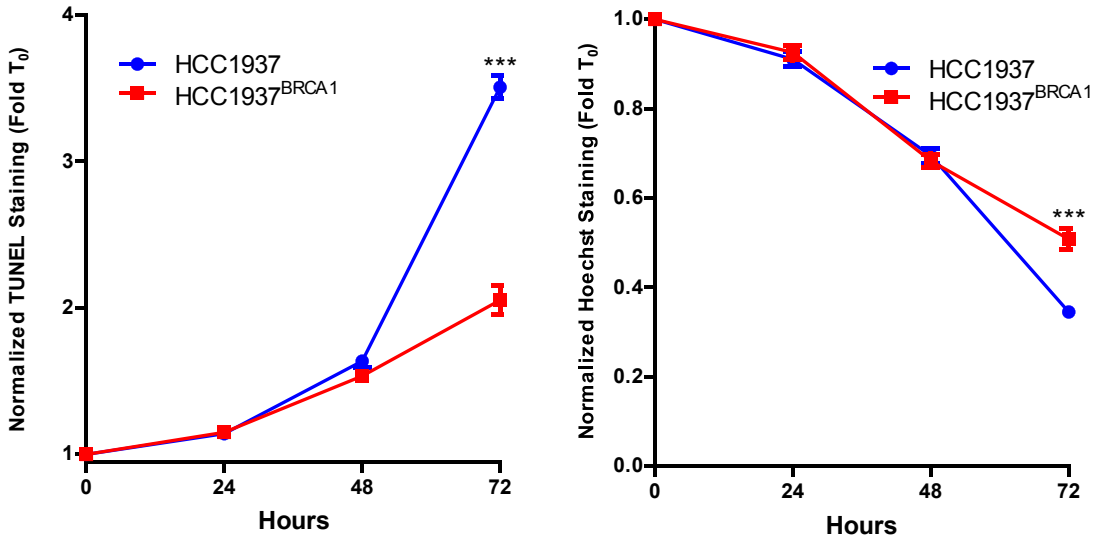

Normalized TUNEL Staining (Fold T0)
HCC1937
HCC1937BRCA1
***
Hours
Normalized Hoechst Staining (Fold T0)
HCC1937
HCC1937BRCA1
***
Hours

Figure 20. (A) High content imaging evaluation of apoptosis and mitotic entry by TUNEL and $pH3^{S28}$ staining, respectively, in HCC1937 and $HCC1937^{BRCA1}$ cells after 24 hours of exposure to 250 nM 17-AAG. (B) Graphical representation of TUNEL and DNA content (Hoechst); error bars represent SEM in three independent experiments. *p<0.05, **p<0.01, ***p<0.001 (student's t-test).

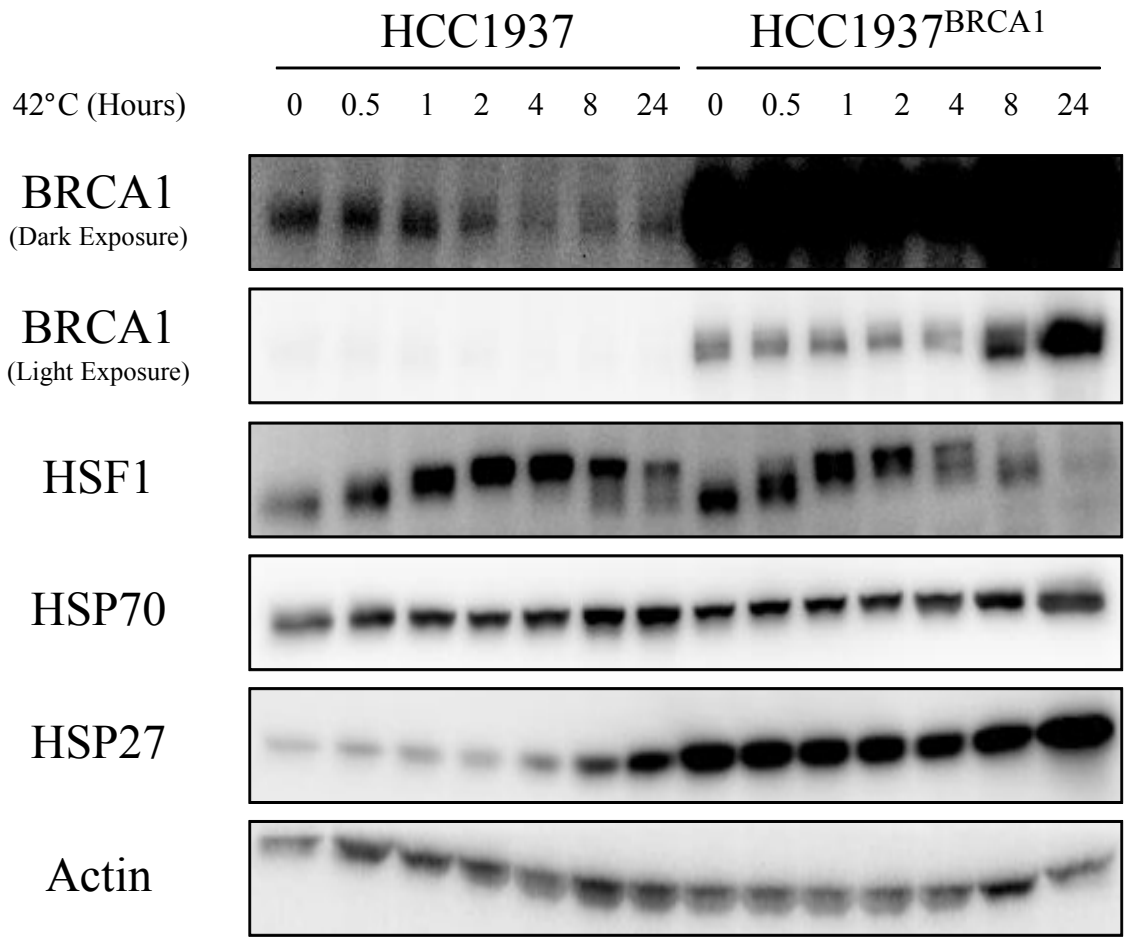

HCC1937
HCC1937BRCA1
42°C (Hours)
0 0.5 1 2 4 8 24 0 0.5 1 2 4 8 24
BRCA1
(Dark Exposure)
BRCA1
(Light Exposure)
HSF1
HSP70
HSP27
Actin

Figure 21. Effect of BRCA1 expression in the heat shock response. HCC1937 and HCC1937^{BRCA1} cells were heat shocked (42°C) for the time indicated and western blots were performed for the indicated antigens.

of heat shock (Figure 21), suggesting that functional BRCA1 may mitigate proteomic stress and prevent heat shock-induced apoptosis.

Discussion

In this chapter, we characterized the role of BRCA1 in regulating 17-AAG-induced cytotoxicity of ionizing radiation and carboplatin, and also evaluated the role of *BRCA1* mutational status in mediating acute cell cycle, replication and apoptotic responses to 17-AAG treatment. As detailed in the previous chapter, 17-AAG treatment abolished BRCA1 expression and almost completely inhibited homologous and non-homologous repair of DSBs. Though BRCA1 is known to be critical for HR, limited data supports a direct role for BRCA1 in NHEJ, though this function remains poorly described and is complicated by conflicting reports suggesting that *BRCA1* mutant cancer cells may rejoin DSBs efficiently (Bau et al., 2004; Merel et al., 2002; Wei et al., 2008a; Zhuang et al., 2006). Our finding that BRCA1 deficiency prevented 17-AAG from further sensitizing cells to IR is intriguing, since NHEJ is thought to repair the bulk of DSBs induced by IR. From these data, we speculate that the radiopotentiating effect of HSP90 inhibitors is due to inhibiting HR alone or that BRCA1 indeed participates in NHEJ.

One report has suggested that BRCA1 is critical for inducing the G_2/M checkpoint in breast cancer cells following treatment with 17-AAG (Zajac et al., 2008). Our data further define the response of both *BRCA1*-mutant and wild-type cells to HSP90 inhibition. Specifically, we show that without functional *BRCA1*, cells experience acute replication stress and progress into mitosis despite the presence of DNA damage. Our data presented in the current and previous chapters strongly suggest that 17-AAG induces loss of BRCA1 expression. We thus modify the

interpretation offered in the aforementioned report and speculate that expression of functional BRCA1 merely delays aberrant mitosis and temporarily mitigates replication stress, and that following 17-AAG-induced BRCA1 degradation, cells with wild-type *BRCA1* will ultimately succumb to the same fate. In direct conflict with our data, this same report indicated no differential sensitivity of *BRCA1*-wild-type and -null cells to 17-AAG. Their study examined the sensitivity of numerous breast cancer cell lines based on *BRCA1* status alone, and did not account for the numerous physiological and genetic differences that can exist between different cell lines. Our examination of sensitivity in a two syngeneic systems with differential expression of BRCA1 revealed a strong correlation between BRCA1 expression and sensitivity to 17-AAG. Our finding that 17-AAG alone induced γH2AX foci formation (and thus accumulation of DSBs) (Chapter II Figure 10) would predict that BRCA1-deficient cells would exhibit increased sensitivity, and our evidence of enhanced synergy between carboplatin and 17-AAG in these cellular models provides further support for this interaction.

Finally, our results suggest that acute inhibition of HSP90 may selectively induce *BRCA1*-mutant or BRCA1-deficient cells to experience replicative stress and/or to enter into aberrant mitosis. BRCA1 expression is strongly induced at the G1/S transition and remains highly expressed through the G2 phase (Blackshear et al., 1998; Chen et al., 1996; Gudas et al., 1996; Rajan et al., 1996; Vaughn et al., 1996). During S phase, BRCA1 localizes to sites of stalled or collapsed replication forks where it interacts with CtIP and the MRE11/RAD50/NBS1 complex to facilitate end resection and lesion bypass through sister chromatid exchange (Scully et al., 1997; Yu et al., 1998). In the absence of functional BRCA1, HR-mediated bypass of stalled replication forks cannot be accomplished, and thus BRCA1-deficient cells rely heavily on ancillary repair

pathways. An interesting and clinically relevant association has been noted between poly(ADP)ribose polymerase (PARP) enzymes and maintenance of replicative potential in *BRCA1*- and *BRCA2*-mutant cells. PARPs are best characterized for their role in base excision repair (BER) of ssDNA breaks, and are required for efficient recruitment of XRCC1 and processing of ssDNA lesions (El-Khamisy et al., 2003; Masson et al., 1998; Sanderson and Lindahl, 2002; Woodhouse et al., 2008). Deficiency of HR (e.g., *BRCA1/2* mutation) renders cells exquisitely sensitive to inhibition of PARP (Farmer et al., 2005; Fong et al., 2009; Fong et al., 2010), and conversely, genetic deficiency of *PARP1* leads to increased frequency of sister chromatid exchange and repair of stalled replication structures by homologous recombination (Oikawa et al., 1980; Simbulan-Rosenthal et al., 1999; Wang et al., 1997). Our finding that 17-AAG selectively reduces replication potential in *BRCA1*-mutant HCC1937 cells suggests that one or more client proteins of HSP90 may have a synthetic lethal relationship with BRCA1. We assume that PARP is not this candidate molecule, as 17-AAG has been demonstrated to be synergistic with PARP inhibiton in glioma cells and treatment with several HSP90 inhibitors induces PARP cleavage (a measure of imminent apoptosis) (Dungey et al., 2009; Khong and Spencer, 2011; Kim et al., 2012; Liu et al., 2011).

As modeled in Figure 22, we propose a model whereby 17-AAG induces accumulation of sporadic DSBs. Cells with functional BRCA1 are able to activate CHK1 in an ATR-dependent manner (Yarden et al., 2002), leading to phosphorylation and degradation of the CDC25A and CDC25C phosphatases (Wagner and Kaufmann, 2010). This in turn leads to S and G2 phase arrest, allowing BRCA1 to facilitate homologous recombination and resolution of spontaneous DSBs. In cells with mutant *BRCA1*, ATR is unable to phosphorylate CHK1, leading to

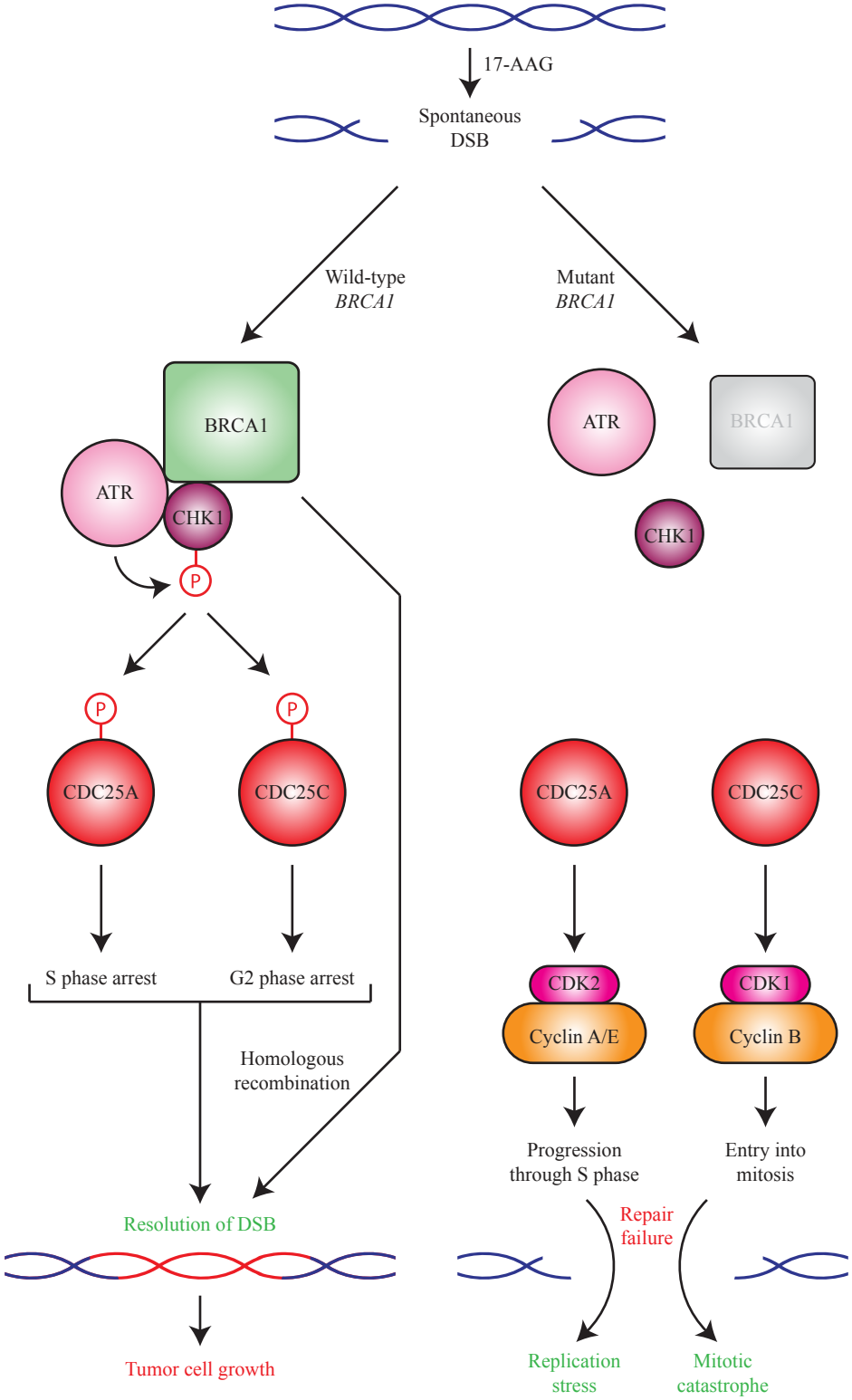

17-AAG
Spontaneous DSB
Wild-type BRCA1
Mutant BRCA1
BRCA1
ATR
CHK1
P
ATR
BRCA1
CHK1
P
P
CDC25A
CDC25C
CDC25A
CDC25C
S phase arrest
G2 phase arrest
CDK2
Cyclin A/E
CDK1
Cyclin B
Homologous recombination
Progression through S phase
Entry into mitosis
Repair failure
Resolution of DSB
Tumor cell growth
Replication stress
Mitotic catastrophe

Figure 22. Model for mechanism by which BRCA1 status regulates acute cell cycle and replication responses to 17-AAG.

accumulation of unphosphorylated CDC25A and CDC25C which will activate the CDK2/Cyclin A/E (S phase) and CDK1/Cyclin B (M phase) cell cycle machinery, respectively. The absence of HR in *BRCA1* mutant cells will also lead to persistence of DSBs, which will trigger replication stress in cells in S phase and ultimately lead to mitotic catastrophe in cells which enter mitosis.

Chapter IV: *BRCA1* Promoter Methylation as a Predictive Biomarker for Response to Adjuvant Anthracycline Therapy in Triple-Negative Breast Cancer

Introduction

Triple-negative (TN) breast cancers (TNBCs) account for approximately 15% of all invasive breast cancers and are typically of high histological grade, demonstrate high mitotic indices, mutations in the *TP53* tumor suppressor gene, and are defined immunohistochemically as those lesions which lack expression of estrogen receptor (ER), progesterone receptor (PR) and HER2. Due to the absence of these receptors, TNBCs are not amenable to the targeted anti-estrogen and anti-HER2 therapies that have dramatically improved survival of patients diagnosed with luminal-type or HER2-positive tumors. Modern pathologic diagnosis continues to rely heavily on expression of ER, PR and HER2, while most breast cancers will not be subjected to molecular taxonomic analysis. Despite the limited use of molecular analysis clinically, several studies have demonstrated that immunohistochemically-defined TNBCs are highly represented in the basal-like breast cancer (BLBC) molecular subtype. Despite the notable similarities between TNBCs and BLBCs, caution should be exercised in equating these classification schemes as numerous studies have demonstrated that not all TNBCs exhibit basal-like transcriptional signatures and not all BLBCs are of the TN immunophenotype (Bertucci et al., 2008; Calza et al., 2006; Cheang et al., 2008; de Ronde et al., 2010; Jumppanen et al., 2007; Nielsen et al., 2004; Rakha et al., 2008a; Rakha and Reis-Filho, 2009; Rakha et al., 2007a; Rakha et al., 2009; Rakha and Ellis, 2009; Rakha et al., 2008b, c; Rakha et al., 2007b; Rouzier et al., 2005; Weigelt et al., 2010).

Loss of normal BRCA1 expression and function has been noted to be among the most common recurrent molecular abnormalities noted in BLBCs. Indeed, tumors arising in *BRCA1* mutation carriers are overwhelmingly triple-negative and exhibit a basal-like transcriptional signature (Lim et al., 2009; Turner and Reis-Filho, 2006). Moreover, sporadic BLBCs commonly

demonstrate downregulated BRCA1 expression in the absence of mutations at the *BRCA1* locus, a phenotype that has been termed "*BRCA*ness" (Thompson et al., 1995; Turner et al., 2004; Turner et al., 2007). As has been discussed extensively in previous chapters, disruption of BRCA1 (or BRCA2) function by genetic or epigenetic mechanisms results in compromised capacity to repair double-strand DNA breaks (DSBs) and interstrand crosslinks (ICLs) by homologous recombination (HR) (Jasin, 2002). While this genomic instability likely underlies the proclivity for tumorigenesis observed in heterozygous individuals, it also may lend to therapeutic exploitation. Agents that induce DSBs (i.e., ionizing radiation and bleomycin) or ICLs (i.e., platinum-based alyklating agents) appear to be significantly more toxic in cells with reduced or absent expression of BRCA1 (Abbott et al., 1999; Bhattacharyya et al., 2000; Husain et al., 1998; Quinn et al., 2003). Accordingly, human breast cancers arising in *BRCA1* mutation carriers are more likely to achieve clinical responses in response to platinum-based agents than non-*BRCA1/2* tumors (Isakoff, 2010). Conversely, because of the critical role of BRCA1 in inducing G2/M arrest in response to microtubule poisons, tumors deficient in BRCA1 tend to be relatively resistant to these agents (Lafarge et al., 2001; Mullan et al., 2001; Quinn et al., 2003; Tassone et al., 2005). Thus, selection of specific cytotoxic agents based on DSB repair capacity may improve responses to traditional chemotherapeutic agents and enable personalized cytotoxic chemotherapy (Price and Monteiro, 2010).

Current first-line treatment for breast cancer is usually anthracycline-based, regardless of immunohistochemical or molecular subtype. While anthracyclines have shown significant activity in breast cancer patients as a collective group, meaningful clinical outcomes in the BLBC subgroup continue to lag behind those that have been achieved in the ER/PR+ and

HER2+ groups. Given the prevalence of BRCA1 dysfunction in TNBCs, it is rational to speculate that agents that induce DSBs or ICLs and require the homologous recombination pathway to be resolved may be more efficacious in treating these lesions. While anthracyclines are known to induce DSBs through inhibition of the re-ligation step of topoisomerase II (Tewey et al., 1984), repair of these lesions does not necessarily require the FA/BRCA pathway, as most DSBs in higher eukaryotes are repaired via non-homologous end joining (NHEJ) (Shrivastav et al., 2008). Since productive resolution of an ICL requires the FA/BRCA pathway, agents which induce ICLs may be especially useful in TNBC treatment.

In this chapter, we retrospectively evaluated whether methylation of a particular CpG island in the *BRCA1* promoter in 39 triple-negative breast cancer patients could predict relapse-free survival (RFS) or overall survival (OS) after adjuvant anthracycline therapy. Methylation of this region of the *BRCA1* gene has been shown in numerous series to be associated with reduced BRCA1 mRNA and/or protein expression (Matros et al., 2005; Scardocci et al., 2006; Wang et al., 2010; Wei et al., 2005). Our data from this this small cohort suggests that methylation of this specific CpG island correlates with reduced BRCA1 mRNA expression and poor RFS and OS in anthracycline-treated patients. We are currently evaluating whether this same biomarker can predict response to neoadjuvant carboplatin in a prospective cohort of TNBC patients.

Clinical Samples and Materials and Methods

Patient Identification

Subjects with early stage (stage I-III) TNBC treated with adjuvant/neoadjuvant chemotherapy between 1996- 2008 at the University of Kansas Medical Center were identified under an IRB

approved protocol and their formalin-fixed paraffin-embedded (FFPE) breast tumor specimens were retrieved from pathology archives.

DNA and RNA Isolation

Tumor-dense (>75%) areas were dissected from paraffin curls (20 μm). Genomic DNA (gDNA) was isolated using the QIAamp FFPE Kit (Qiagen) and total RNA was isolated using the RecoverAll® Kit (Ambion/Life Technologies).

Bisulfite Conversion and Methylation-Specific PCR

Tumor-derived gDNA was bisulfite converted using the EpiTect® Plus FFPE Bisulfite Kit (Qiagen, Valencia, CA). Purified converted DNA was subjected to methylation-specific PCR (MSP) using the EpiTect® MSP Kit. The locus being analyzed (Figure 1) and primer sets have been previously validated (Esteller et al., 2000; Matros et al., 2005; Scardocci et al., 2006; Wang et al., 2010; Wei et al., 2005):

BRCA1 Unmethylated Forward	5' – TTGGTTTTTGTGGTAATGGAAAAGTGT – 3'
BRCA1 Unmethylated Reverse	5' – CAAAAAATCTCAACAAACTCACACCA – 3'
BRCA1 Methylated Forward	5' – TCGTGGTAACGGAAAAGCGC – 3'
BRCA1 Methylated Reverse	5' – AAATCTCAACGAACTCACGCCG – 3'

All primers were diluted to approximately 300 nM and all reactions used $T_m = 55°C$. Specificity of reactions was confirmed using EpiTect® Universal Bisulfite-Converted DNA (Qiagen). Amplified samples were electrophoresed on a 2.5% agarose gel, stained with ethidium bromide, and visualized on a UVP Bioimaging System (UVP).

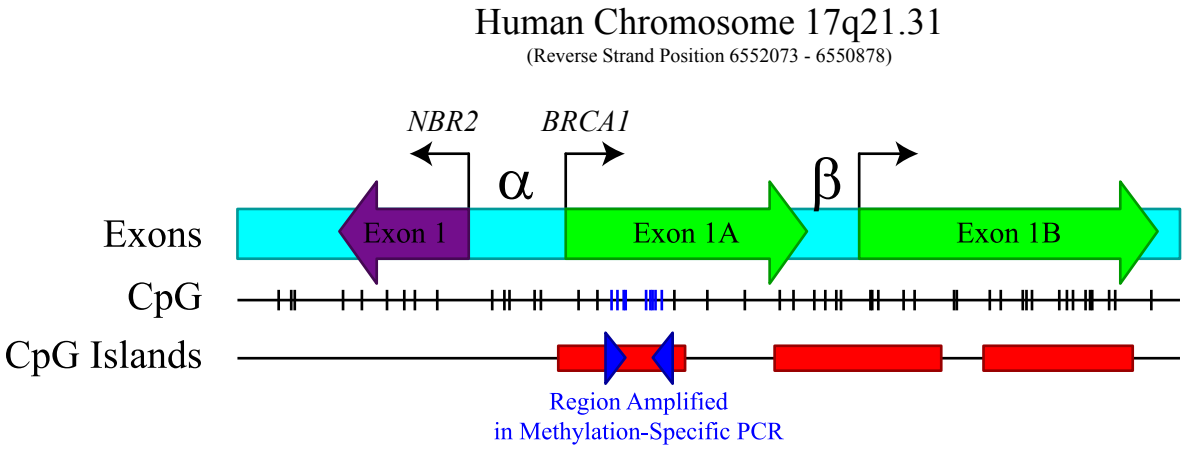

Human Chromosome 17q21.31
(Reverse Strand Position 6552073 - 6550878)
NBR2
BRCA1
α
β
Exons
Exon 1
Exon 1A
Exon 1B
CpG
CpG Islands
Region Amplified
in Methylation-Specific PCR

Figure 1. Diagrammatic representation of the *BRCA1* promoter locus amplified by methylation-specific PCR. *NBR2* is the "neighbor of *BRCA1* gene 2" ORF. a and b are the two promoter of the human *BRCA1* gene, and a bidirectionally regulates the *NBR2* gene. CpG islands were predicted by MethPrimer (Li Lab, UCSF) using observed/expected ration > 0.6 and %GC > 50%. The region amplified in our assay is denoted by blue arrowheads.

Quantitative Real-Time PCR

Tumor-derived RNA was converted to cDNA and subjected to TaqMan® RT-PCR using probes specific for BRCA1 (Hs00173233_m1) and cyclophilin A (Hs99999904_m1) (Applied Biosystems/Life Technologies). Each sample was assayed in duplicate for both targets on an ABI 7500 RT-PCR thermal cycler (Applied Biosystems/Life Technologies). BRCA1 transcript levels were normalized to cyclophilin A using the ΔΔCt method (Livak and Schmittgen, 2001).

Statistics

Relapse free survival (RFS) and overall survival (OS) were estimated according to the Kaplan-Meier method and compared among groups with log-rank statistic. Cox proportional hazards models were fit to determine the association of *BRCA1* promoter methylation with the risk of recurrence and death after adjustment for other characteristics.

Results

Assay Performance and Subject Demographics

Archived tumor tissue was identified for 39 subjects. *BRCA1* promoter methylation-specific PCR (MSP) was successful in 37/39 (95%) of specimens and BRCA1 mRNA qRT-PCR was successful in 35/39 (90%) of specimens. Table 1 summarizes the demographics of the 37 patients for whom MSP analysis was successful. Consistent with current clinical practice, the majority (90%) of these patients had received an anthracycline as first-line chemotherapy (Table I).

BRCA1 Promoter Methylation, mRNA Expression and Clinical Outcomes

Median Age at Diagnosis (Range)	52 Years (33-80)
Ethnicity	
Caucasian	27 (74%)
African	7 (18%)
East Asian	3 (8%)
Lymph Node Status	
Negative	18 (49%)
Positive	19 (51%)
Stage	
I	11 (30%)
II	14 (38%)
III	12 (32%)
Neo/Adjuvant Chemotherapy	100%
Anthracycline	33 (90%)
Taxane	27 (74%)
Platinum	5 (13%)
BRCA1 Promoter Methylation	
Negative	26 (70%)
Positive	11 (30%)

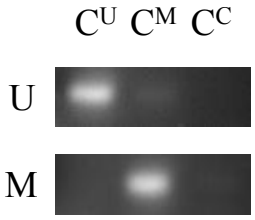
Table 1. Patient demographics and tumor and therapy characteristics.

Of the 37 tumors in which the MSP assay was successful, 11 (30%) exhibited *BRCA1* promoter methylation (Figure 2A-B). Methylation of the *BRCA1* gene was significantly associated with downregulation of BRCA1 mRNA expression (Figure 3), but we did not uncover any significant association with age, nodal status or T stage. At a median follow-up of six years (8-148 months), there have been 19 (51%) recurrences and 14 (37%) deaths. On univariate analysis, node positivity, higher stage and presence of *BRCA1* promoter methylation were associated with poorer RFS and OS (Figure 4A-B). Five-year RFS and OS was 27% and 36%, respectively, for patients with methylation of the *BRCA1* promoter compared to 61% and 81%, respectively, for patients whose tumors did not exhibit *BRCA1* promoter methylation. After adjustment for stage and nodal status, patients with *BRCA1* promoter methylation still exhibited poorer OS compared to patients without methylation (HR=3.0, 95% CI: 1.1-8.0, p=0.002).

Discussion

Numerous studies have evaluated clinical outcomes in *BRCA1*-associated breast cancer, and a recent large meta-analysis argues that the prognosis of both *BRCA1*- and *BRCA2*-associated breast cancers is similar to that of sporadic breast cancers (Bordeleau et al., 2010). This is in stark contrast to a large body of evidence suggesting that *BRCA1*- and *BRCA2*-associated epithelial ovarian cancer carries an improved prognosis when compared to sporadic cancers (Bolton et al., 2012). As was discussed in Chapter I, platinum-based chemotherapeutic agents have been a mainstay of treatment for epithelial ovarian cancer for decades, while first-line use of these agents in breast cancer is uncommon. *BRCA1/2*-mutant cells should exhibit intrinsic sensitivity to platinum-based agents, as resolution of platinum-induced damage requires homology-directed repair of interstrand crosslinks. It is thus interesting to speculate that use of

A


C^U C^M C^C
U
M

B


U M U M U M U M U M
9609746 0502413 0404064 9609289 0410539
0203016 0108896 0401383 0309614 0208054
9901907 0804685 9807620 0509208 0409942
0203772 9706380 0406517 0209773 0400800
9608033 0400055 0103083 9902100 0406550
0605753 0202859 0601557 0500388 0612598
0410445 0506172 0003094 0509808 0410160
0501511 0903932 0501672 0206254 0409539

Figure 2. (A) Specificity controls for the *BRCA1* promoter methylation-specific PCR reaction. Unconverted genomic DNA (C^C), universally unmethylated bisulfite-converted genomic DNA (C^U) and universally methylated bisulfte-converted genomic DNA (C^M) were amplified with primers specific for the bisulfite-converted unmethylated (U) or methylated (M) *BRCA1* promoter. (B) Methylation-specific PCR of the *BRCA1* promoter from human triple-negative breast cancer patients. Seven digit number indicates surgical pathology specimen identification number, U and M denote reactions conducted with the unmethylated and methylated *BRCA1* promoter primers, respectively. Samples noted in red and blue were judged to be unmethylated and methylated, respectively. Samples noted in grey failed PCR amplification.

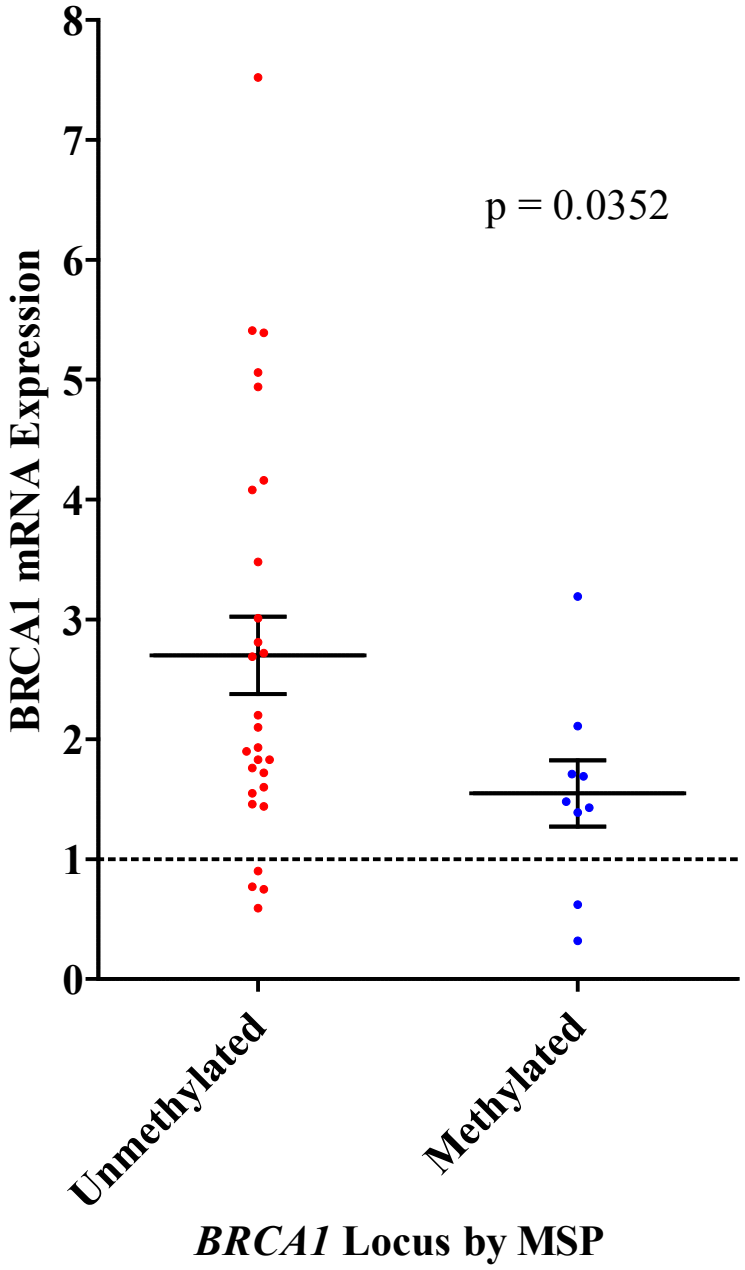
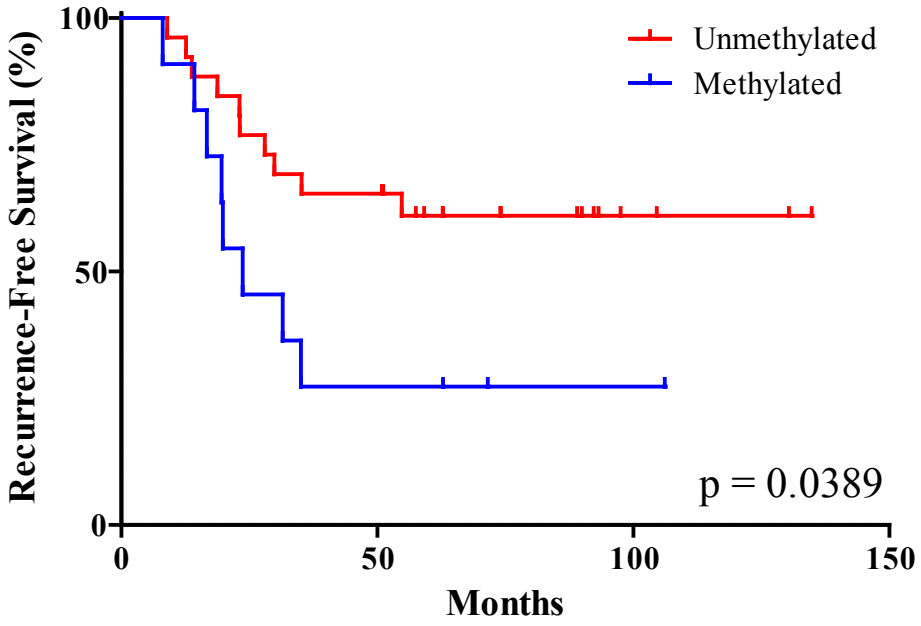

8
7
6
5
4
3
2
1
0
BRCA1 mRNA Expression
p = 0.0352
Unmethylated
Methylated
BRCA1 Locus by MSP

Figure 3. Association between BRCA1 promoter methylation and BRCA1 mRNA expression. Significance calculated by Mann-Whitney test.

A


Recurrence-Free Survival (%)
100
50
0
0
50
100
150
Months
Unmethylated
Methylated
p = 0.0389

B

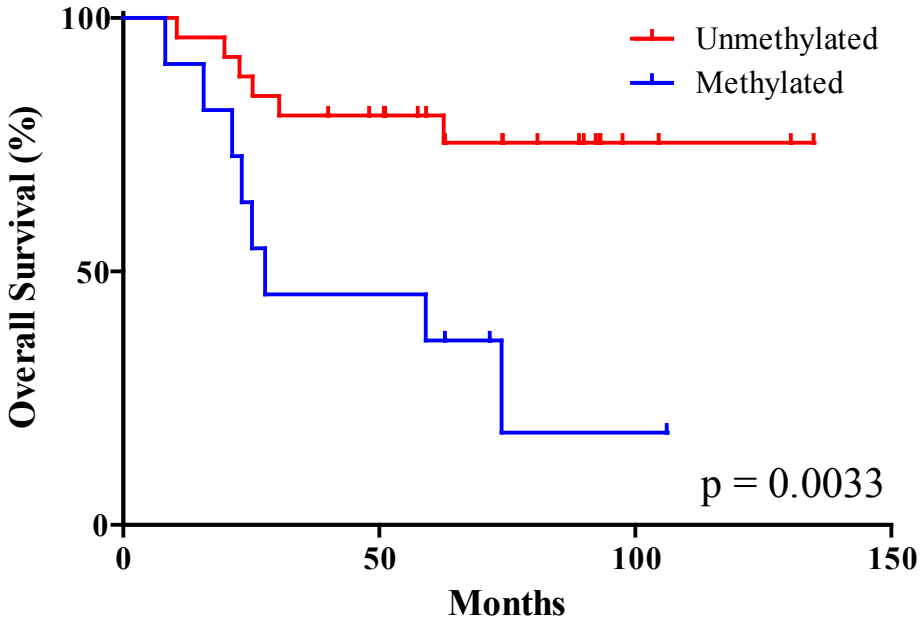

Overall Survival (%)
100
50
0
0
50
100
150
Months
Unmethylated
Methylated
p = 0.0033

Figure 4. Kaplan-Meier relapse-free survival (A) and overall survival (B) curves based on BRCA1 methylation status. Significance calculated by log-rank test.

platinum agents in *BRCA1/2*-associated breast cancers may render a similar trend to that seen in ovarian cancer.

In sporadic ovarian cancers, deficiency of BRCA1/2 expression or function (i.e., "*BRCA*ness") has also been associated with improved responses and/or prolonged survival to platinum-based chemotherapy (Konstantinopoulos et al., 2010; Quinn et al., 2007; Swisher et al., 2009; Taniguchi et al., 2003; Teodoridis et al., 2005; Weberpals et al., 2009). In sporadic breast cancers, it has been noted by several studies that methylation of *BRCA1* may be associated with poor clinical outcome (Chen et al., 2009; Sharma et al., 2009; Xu et al., 2009; Xu et al., 2010). Again, we speculate that the absence of a survival benefit in sporadic breast cancers with *BRCA*ness may be attributable to the omission of a platinum-based chemotherapeutic agent in the first-line treatment of these neoplasms. This is supported by a recent study which used a comparative genomic hybridization (CGH) signature derived from *BRCA1*-mutant breast cancers to define sporadic HER2-negative tumors as *BRCA1*-like or non-*BRCA1*-like. This report noted that patients with the *BRCA1*-like CGH profile experienced improved response and clinical outcomes on high-dose platinum-based chemotherapy compared to standard anthracycline-based chemotherapy (Vollebergh et al., 2011). Similar results were obtained in a study of a multiplex methylation-based *BRCA*ness profile in which high-dose alkylative chemotherapy including carboplatin led to improved survival in patients with a *BRCA*ness profile (Lips et al., 2011).

Our study defines *BRCA1* promoter methylation as an adverse prognostic biomarker for anthracycline-treated TNBC. These data, taken with the immense body of evidence supporting the use of platinum-based agents in ovarian cancer, suggest that use of platinum in TNBCs with

documented *BRCA1/2*-mutation or somatic loss of BRCA1 expression or function may improve clinical outcomes. In addition to the potential improvement in anti-neoplastic activity, avoidance of anthracyclines in patients with germline *BRCA1/2* mutation may be clinically important in its own rite, as myocardium-specific knockout of *Brca1* or *Brca2* in mice exaggerated cardiac dysfunction after myocardial infarct and, importantly, increased anthracycline-induced cardiac toxicity (Shukla et al., 2011; Singh et al., 2012). Whether this hypersensitivity exists in the haploinsufficient myocardium of *BRCA1* and *BRCA2* patients remains to be studied. In addition to the small number of samples evaluated, a major limitation of our study is that we were unable to ascertain *BRCA1*/2 mutation status, as only fixed paraffin-embedded tissue was available for analysis. Despite this shortcoming, we can infer that all of the *BRCA1*-methylated tumors were from patients with wild-type *BRCA1/2*, as genetic and epigenetic inactivation of BRCA1 is virtually mutually exclusive in both breast and ovarian cancer (TCGA, 2011; Vollebergh et al., 2011; Yang et al., 2011).

We are currently examining *BRCA1* promoter methylation prospectively in a cohort of 30 neoadjuvant platinum-treated TNBC patients, all of whom have undergone comprehensive *BRCA1* and *BRCA2* germline mutation testing. We expect that this study will illuminate BRCA1 as a critical determinant of platinum sensitivity in TNBC and will lead to a pilot trial in which patients receive tailored cytotoxic chemotherapy based on BRCA1-dependent DNA damage repair capacity.

Chapter V: Perspective and Conclusions

Intrinsic and acquired therapeutic resistance remains a tremendous clinical obstacle in the treatment of both breast and ovarian cancer. A comprehensive understanding of the mechanisms which underlie therapeutic failure, coupled with advances in genetic and pharmacologic interventions which can combat resistance are certain to improve clinical outcomes for breast and ovarian cancer patients.

BRCA1-Dependent DNA Damage Responses and Efficacy of Chemo- and Radiotherapy

Breast cancer patients with no evidence of metastatic disease (i.e., stages I-III) are treated locally with curative intent by surgical excision and adjuvant radiotherapy. Though such early stage disease is defined by the absence of overt metastatic involvement, the existence of micrometastatic deposits is likely in many of these patients. To avoid recurrence from and progression of micrometastases, adjuvant systemic treatments including chemotherapy, endocrine therapies and biologic agents are utilized in an attempt to eradicate disease that was not surgically excised or biologically sterilized with radiotherapy. In patients with more advanced, but still potentially curable disease (e.g., stage IIIB and operable stage IIIC), such systemic therapies may be used in the neoadjuvant setting to reduce tumor burden followed by surgery and radiotherapy.

Treatment with ionizing radiation (IR) is an integral component of breast cancer therapy for patients with both early stage and advanced disease. Nearly all patients who undergo breast conserving surgery (i.e., lumpectomy) for invasive and *in situ* disease will receive radiation, as will many patients undergoing mastectomy (Carlson and McCormick, 2005; Recht et al., 2001). In both cohorts, the addition of IR has been shown to dramatically reduce local recurrence,

which has been independently associated with improving overall survival (Clarke et al., 2005; Gebski et al., 2006). In patients with advanced and metastatic disease, radiation is indispensable in slowing progression and providing palliation (Rades et al., 2006; van der Linden et al., 2005; Wadasadawala et al., 2007). The lethal effects of IR are due to production of unrepairable DNA lesions involving DNA double-strand breaks (DSBs), and repair of DSBs is a major mechanism by which normal and cancer cells become resistant to IR.

It has been noted that radiation produces fewer γH2AX foci (the hallmark of DSB induction) in a therapy-resistant population of quiescent breast cancer cells commonly known as cancer stem cells (CSCs), and that such foci resolved faster (i.e., were repaired more efficiently) in CSCs than in non-CSC populations (Diehn and Clarke, 2006; Phillips et al., 2006; Woodward et al., 2007). Non-homologous end joining (NHEJ), a relatively error-prone process that directly ligates strands following a break, is responsible for the bulk of DSB repair following irradiation, while the error-free homologous recombination (HR) pathway, involving BRCA1, BRCA2 and RAD51, plays a modest role in repair of IR-induced lesions. Interestingly, it has been noted from *in vitro* studies of human breast cancer cell lines that HR, and not NHEJ, appears to be hyperactive in breast CSCs and may be responsible for the intrinsic radiation resistance of these cells (Yin and Glass, 2011). As is the case for radiotherapy, heightened BRCA1-dependent DNA damage responses after neoadjuvant chemotherapy have been noted to predict poorer therapeutic response in breast cancer patients (Asakawa et al., 2010; Graeser et al., 2010). Thus, targeting BRCA1-dependent DNA damage repair may represent a plausible mechanism to overcome radiation and chemotherapeutic resistance in breast cancer.

For ovarian cancer, cytoreductive "debulking" surgery followed by platinum-based chemotherapy remains the mainstay of first-line treatment (Schwartz, 2008; Wakabayashi et al., 2008). Because epithelial ovarian cancers are most commonly diagnosed at advanced stages (III or IV), most patients relapse and succumb to progressive disease despite extensive cytoreduction and often complete clinical responses to adjuvant chemotherapy (Miller and Rustin, 2010). A major determinant of clinical outcome following relapse is the platinum-free interval (PFI), with women experiencing a recurrence within the first six months after completion of primary therapy defined as platinum-resistant, and women who experience a recurrence after six months being termed platninum-sensitive. The former group of patients is treated in the second-line setting with non-cross-resistant agents, commonly including pegylated liposomal doxorubicin (PLD) while those who experience a remission lasting longer than six months may benefit from additional cycles of platinum-containing chemotherapy (Cantu et al., 2002; Monk and Coleman, 2009; Pujade-Lauraine and Alexandre, 2011). These studies argue that preserving or extending platinum-sensitivity in both primary and recurrent epithelial ovarian cancers are likely to improve survival.

Recurrent ovarian cancers exhibit dramatically enhanced interstrand crosslink repair capacity (Wynne et al., 2007), suggesting that hyperactivity of BRCA1-dependent DNA damage repair is a significant contributor to therapeutic resistance in this setting. This is supported by reports that high expression of BRCA1 or frame-restoring mutations in recurrent *BRCA1*-associated ovarian cancers is associated with platinum resistance and poor clinical outcome (Konstantinopoulos et al., 2010; Quinn et al., 2007; Swisher et al., 2009; Swisher et al., 2008; Teodoridis et al., 2005; Weberpals et al., 2009).

The purpose of this study was to evaluate the potential use of the clinically-relevant HSP90 inhibitor 17-AAG in preventing BRCA1-dependent DNA damage repair, and to uncover the link between epigenetic silencing of *BRCA1* and response to anthracycline chemotherapy in triple-negative breast cancer. During the course of these studies, we have identified a novel biological interaction between BRCA1 and HSP90 and demonstrate *in vitro* that 17-AAG can destabilize BRCA1, and in effect, abolish BRCA1-dependent DNA damage repair. Importantly, we also demonstrate that 17-AAG is able to resensitize both sporadic and hereditary breast and ovarian cancer cell lines to ionizing radiation and/or platinum in a BRCA1-dependent manner. These findings may have significant implications for treating platinum-resistant or -refractory ovarian cancer, and further argue that platinum-containing regimens may be superior to anthracycline therapy for a significant proportion of triple-negative breast cancer patients.

Chapter II: BRCA1 Stability and Function is Regulated by HSP90

HSP90 inhibitors are known to increase sensitivity to numerous genotoxic agents, including radiation and platinum-based crosslinking agents (Arlander et al., 2003; Bagatell et al., 2005; Camphausen and Tofilon, 2007; Dote et al., 2006; Georgakis et al., 2006; Munster et al., 2001; Yao et al., 2007). Despite this observation, the mechanism(s) by which these agents enhance therapeutic efficacy remain incompletely understood. The aim of this study was to evaluate whether HSP90 inhibitors influenced the expression and/or function of BRCA1, a critical determinant of DSB and ICL repair capacity. We demonstrate that the clinically-relevant HSP90 inhibitor 17-AAG induces polyubiquitination and proteasome-mediated degradation of BRCA1 in numerous cancer cell lines and in effect, abolishes localization of BRCA1 to sites of DNA

damage. We also functionally document dramatic reduction in repair of DSBs by both homologous recombination and non-homologous end joining after 17-AAG treatment. Other components involved in the repair of DSBs and ICLs by homologous recombination are known clients of HSP90, including FANCA, CHK1 and BRCA2 (Arlander et al., 2003; Noguchi et al., 2006; Oda et al., 2007). Despite these known connections, BRCA1 occupies an apical position in the HR pathway and is epistatic to the function of all three of these proteins (Bhattacharyya et al., 2000; Folias et al., 2002; Yarden et al., 2002; Zhang et al., 2009). Our data thus suggest that 17-AAG-induced loss of BRCA1 is a key upstream event leading to DSB repair failure.

Chapter III: BRCA1 Regulates 17-AAG-Induced Radio- and Chemosensitization, G2/M Checkpoint Activation, Replication Stress Response and Apoptosis

In addition to coordinating the repair of DSBs and ICLs, BRCA1 also plays critical roles in activating cell cycle checkpoints after genotoxic stress, maintaining the integrity of replication forks and regulating damage-associated apoptosis (Powell and Kachnic, 2003; Quinn et al., 2003; Scully et al., 1997; Thangaraju et al., 2000; Tibbetts et al., 2000; Yarden et al., 2002). Given our finding that 17-AAG can regulate BRCA1 expression and function, the studies in this chapter were designed to evaluate whether 17-AAG differentially affected sensitivity to cytotoxic agents or influenced acute cell cycle, DNA replication and apoptotic responses in cells expressing mutant or wild-type BRCA1. Towards this end, we demonstrated that BRCA1-deficient and *BRCA1*-mutant breast cancer cells are hypersensitive to 17-AAG, and that 17-AAG was unable to hypersensitize BRCA1-deficient cells to ionizing radiation. These findings are consistent with our assertion that 17-AAG-induced BRCA1 degradation is an important mediator of therapeutic hypersensitivity after HSP90 inhibition. Furthermore, we show that 17-AAG can

sensitize *BRCA1*-mutant and sporadic breast and ovarian cancer cells to carboplatin, and that carboplatin resistance imbued by complementation of wild-type *BRCA1* can be abolished by 17-AAG treatment. Our data also show that *BRCA1*-mutant breast cancer cells enter into catastrophic mitosis, acutely lose replication capacity, and undergo apoptosis following 17-AAG treatment, while an isogenic cell line complemented with wild-type *BRCA1* is able to arrest in G2, maintain DNA synthesis and delay apoptosis. Collectively, these data suggest that 17-AAG may be useful in treating breast and ovarian cancers which lack functional BRCA1 by two discrete mechanisms. First, since 17-AAG causes the accumulation of spontaneous DSBs, cells which lack BRCA1 should be intrinsically hypersensitive to 17-AAG. Second, and perhaps more importantly, 17-AAG can destabilize mutant and wild-type BRCA1, and should therefore be effective in preventing and reversing BRCA1-dependent DSB/ICL repair-mediated resistance. Since augmented DNA repair appears to be a significant contributor to radiation and platinum resistance, we speculate that HSP90 inhibitors may find success in treating resistant or refractory breast and ovarian cancer.

Chapter IV: *BRCA1* Promoter Methylation as a Predictive Biomarker for Response to Adjuvant Anthracycline Therapy in Triple-Negative Breast Cancer

Triple-negative breast cancer (TNBC) is an aggressive form of breast cancer, and effective treatment of TNBC is complicated by the paucity of targeted therapies. Recent insights into the molecular pathogenesis of TNBCs have revealed that defects in homologous recombination, especially associated with the *BRCA1* gene, are common and may be causally associated with the pathogenesis of these lesions. Numerous studies have shown that ovarian cancers with inherited or acquired defects in BRCA1 expression or function may enjoy improved responses and/or

clinical outcomes after platinum-based adjuvant cytotoxic therapy. Similar trends have not emerged in breast cancer. Platinum-based chemotherapeutic agents induce ICLs, a DNA lesion that requires BRCA1 to be functionally resolved. It is thus not unexpected that BRCA1-deficient tumors should exhibit hypersensitivity to platinum, and repair-failure of platinum-induced lesions in BRCA1-deficient ovarian cancer almost certainly underlies the improved responses in these lesions. It is thus interesting to speculate that improved survival in BRCA1-deficient breast cancer has not emerged because anthracyclines, rather than platinum, are used in the first-line treatment of most breast cancer patients. We sought to understand whether methylation of the *BRCA1* promoter could predict responses to adjuvant anthracycline-based chemotherapy in a cohort of 39 TNBC patients. Methylation of *BRCA1* was associated with reduced expression of BRCA1 mRNA, and significantly poorer relapse-free and overall survival, even after adjustment for stage, nodal status. This finding is not unexpected, as tumor cells lacking BRCA1 have enhanced proliferation rates and may be more invasive (Coene et al., 2011; Thompson et al., 1995; Wang et al., 2008; Yasmeen et al., 2008). Based on our work and the work of others, we argue that platinum-based chemotherapeutic agents should exploit an intrinsic vulnerability of BRCA1-deficient TNBCs and that ascertainment of ICL repair capacity, and subsequent tailoring of cytotoxic chemotherapy may improve clinical outcomes and survival in TNBC. To address this issue, we are currently examining *BRCA1* promoter methylation in a prospective cohort of TNBC patients treated with neoadjuvant carboplatin.

Perspective

Genetic and/or epigenetic inactivation of the FA/BRCA pathway, whether inherited or acquired, incites genomic instability and contributes to tumor initiation and progression. While loss of

normal DSB/ICL repair capacity can be causally associated with cancer pathogenesis, the notion that dysfunction and hyperfunction of DNA repair pathways are both rational targets in anti-neoplastic therapy is gaining momentum. In this thesis, we have identified and mechanistically characterized a "druggable" interaction between BRCA1 and HSP90 that, when pharmacologically manipulated with a clinically-viable small molecule, can dramatically alter the ability of breast and ovarian cancer cells to recover after DSB/ICL induction. This finding is of substantial clinical importance, as it is now appreciated that tumors with high expression of BRCA1 respond poorly to key classes of chemotherapeutic agents. Our data indicate that HSP90 inhibitors can destabilize BRCA1, and in effect, abolish BRCA1-dependent repair-mediated resistance. We also provide compelling evidence that epigenetic inactivation of BRCA1 in aggressive TNBCs predicts poor response to a standard chemotherapeutic regimen used in breast cancer therapy, and that implementation of alternative agents which exploit the intrinsic vulnerability of BRCA1-deficient cells may lead to improved clinical outcomes. Ultimately, this composition illuminates homologous recombination as a duplicitous process; in normal cells, HR is a custodian of genomic integrity and antagonizes malignant transformation, while in a cancer cell, this same pathway confers a sinister resistance that hampers the efficacy of therapy.

Chapter VI: References

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Appendix A: Drug Synergy Calculations

The following has been adapted from the manual for CalcuSyn (Chou and Hayball, 1997).

For the median-effect equation:

$$D = D_m \left[\frac{f_a}{1 - f_a}\right]^{\frac{1}{m}}$$

[Eq. 1]

where D is the dose of drug, D_m is the median-effect dose signifying potency, f_a is the fraction of cells affected by dose D, and m is an exponent signifying the sigmoidicity of the dose-effect curve. D_m can be readily determined by calculating the x-intercept of the median-effect plot, according to the graphical representation of:

$x = \log_{10}[D]$ versus $y = \log_{10}\left[\frac{f_a}{1-f_a}\right]$

[Eq. 2]

The slope of the median-effect plot will yield m, and $m = 1$ if the dose-effect curve is hyperbolic, $m > 1$ for sigmoidal curves, and $m < 1$ for negative-sigmoidal curves.

Equation 1 may be linearized to give:

$$\log_{10}\left[\frac{f_a}{1 - f_a}\right] = m \log_{10}[D] - m \log_{10}[D_m]$$

[Eq. 3]

Substituting:

$$x = \log_{10}[D]$$

[Eq. 4]

$$y = \log_{10}\left[\frac{f_a}{1 - f_a}\right]$$

[Eq. 5]

$$Y_{int} = -m \log_{10}[D_m]$$

[Eq. 6]

Equation 3 can be represented by the straight-line equation:

$$Y = mx + Y_{int}$$

[Eq. 7]

Linear regression of Equation 4 produces non-independent parameters Y_{int} (the y-intercept) and m (the slope), and the statistical relationship between these two parameters is the correlation coefficient $CCSI$:

$$CCSI = -\overline{\log_{10}[D]}\left[\frac{SE_m}{SE_{Y_{int}}}\right]$$

[Eq. 8]

By rearranging Equation 6, $\log_{10}[D_m]$ can be derived as:

$$\log_{10}[D_m] = -\frac{Y_{int}}{m}$$

[Eq. 9]

Assuming the errors of Y_{int} and m are normally distributed, the parameter $\log_{10}[D_m]$ must also be normally distributed according to:

$$SE[\log_{10}[D_m]] = \log_{10}[D_m] \sqrt{\left(\frac{SE[Y_{int}]}{Y_{int}}\right)^2 + \left(\frac{SE[m]}{m}\right)^2 + 2CCSI\frac{SE[Y_{int}]SE[m]}{Y_{int}m}}$$

[Eq. 10]

Similarly, the error in D can be can be derived by a similar treatment. Rearranging Equation 3 to yield:

$$\log_{10}[D] = \frac{1}{m}\left[\log_{10}\left[\frac{f_a}{1-f_a}\right] - Y_{int}\right]$$

[Eq. 11]

lends to an estimation in the error of $\log_{10}[D]$ of:

$$SE[\log_{10}[D]]$$

$$= \log_{10}[D] \sqrt{\left(\frac{SE[Y_{int}]}{\log_{10}\left[\frac{f_a}{1-f_a}\right] - Y_{int}}\right)^2 + \left(\frac{SE[m]}{m}\right)^2 + 2CCSI\frac{SE[Y_{int}]SE[m]}{\log_{10}\left[\frac{f_a}{1-f_a}\right] - Y_{int}m}}$$

[Eq. 12]

To evaluate the combinatorial effect of two drugs, the combination index CI for a given effect can be represented by:

$$CI_{A+B} = \frac{D_{\frac{A}{A+B}}}{D_A} + \frac{D_{\frac{B}{A+B}}}{D_B} + \alpha\frac{D_{\frac{A}{A+B}}D_{\frac{B}{A+B}}}{D_A D_B}$$

[Eq. 13]

where $\alpha = 1$ for a mutually non-exclusive case and $\alpha = 0$ for a mutually exclusive case, and D_A refers to the dose of drug A alone required to manifest a given effect and $D_{\frac{A}{A+B}}$ refers to the dose of drug A in combination with drug B which gives an equal effect.

If the ratio of quantities of each drug is constant across a range of doses, then:

$$D_{\frac{B}{A+B}} = \frac{[B]}{[A]}D_{\frac{A}{A+B}}$$

[Eq. 14]

Thus, for constant ratio experimental parameters, Equation 13 can be expressed as:

$$CI_{A+B} = D_{\frac{A}{A+B}}\left[\frac{1}{D_A} + \frac{[B]}{[A]D_B} + \alpha\frac{[B]D_{\frac{B}{A+B}}}{[A]D_A D_B}\right]$$

[Eq. 15]

Algebraic estimation of the uncertainty in CI makes the assumption that the uncertainty is normally distributed. Since the doses of drugs A and B are logarithmically distributed, this method is not ideal, but such estimation appears to be a fairly valid assumption and avoids the tedious process of calculating the uncertainty in CI using iterative random sampling. The standard deviation of the dose of drug is estimated as:

$$SE[D] = \frac{\left(10^{(\log_{10} D + SE[\log_{10} D])} - 10^{(\log_{10} D - SE[\log_{10} D])}\right)}{2}$$

[Eq. 16]

and thus the approximated error in CI for mutually exclusive cases (i.e., $\alpha = 0$) becomes:

$$SE(CI_{A+B}) = \sqrt{\left\{\frac{D_{\frac{A}{A+B}}}{D_A}\left[\frac{SE\left[D_{\frac{A}{A+B}}\right]}{D_{\frac{A}{A+B}}} + \frac{SE[D_A]}{D_A}\right]\right\}^2 + \left\{\frac{D_{\frac{B}{A+B}}}{D_B}\left[\frac{SE\left[D_{\frac{B}{A+B}}\right]}{D_{\frac{B}{A+B}}} + \frac{SE[D_B]}{D_B}\right]\right\}^2}$$

[Eq. 17]

When $CI = 1$, combinatorial drug effects are additive, and the manifested activity of one drug is independent of the activity of the other drug in the combination. For $CI < 1$, the net effect of the two drugs in combination is greater than the sum of the expected effect of both drugs alone at their equivalent concentrations, thus inferring a synergistic relationship between drugs A and B. When $CI > 1$, the combination of drugs A and B produces an effect that is less than the sum of the expected effect of both drugs alone at their equivalent concentrations, suggesting antagonism between drugs A and B. The CI is a widely accepted quantifier of combinatorial drug interactions (Chou and Talalay, 1983, 1984).